1-1-2012

Allosteric Modulation of Metabotropic Glutamate Receptor 5 as a Treatment for Pain

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by

Michael Christopher Montana

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

May 2012

Saint Louis, Missouri

ABSTRACT OF THE DISSERTATION

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Metabotropic glutamate receptor 5 (mGlu5) has been suggested to play a role in the development and maintenance of chronic pain. mGlu5 is expressed at synapses throughout the pain neuraxis where it is believed to modulate the function of ion channels that underlie nociceptive transduction and transmission. Injections of mGlu5 agonists cause hypersensitivity or nocifensive behavior when administered peripherally, intrathecally, and centrally. In addition, pharmacological antagonism of mGlu5 has been suggested to be analgesic in a variety of animal pain models. Unfortunately, the selectivity of antagonists used in these studies has been called into question, suggesting that at least some of the analgesic properties of putatively selective mGlu5 antagonists may be due to off-target effects. Despite a wealth of data supporting the targeting of mGlu5 to treat pain, both antagonist selectivity issues and the lack of an mGlu5 antagonist approved for use in humans have hindered testing beyond the preclinical stage. To address this issue I assessed the pain behavior of mice with a genetic absence of

mGlu5. mGlu5 KO mice exhibit pain-like behaviors, however the duration and intensity of these behaviors are attenuated in multiple nociceptive tests, including spontaneous nociceptive behavior in the formalin test, and mechanical hypersensitivity induced by Complete Freund's Adjuvant. In addition, I assessed the analgesic properties of fenobam, [N-(3-chlorophenyl)-N'-(4,5-dihydro-1-methyl-4-oxo-1H-imidazole-2-yl)urea], an anxiolytic recently identified as a selective and potent non-competitive negative allosteric modulator of mGlu5, and previously shown to be safe and effective in clinical trials of human anxiety. I found Fenobam to be analgesic in models of chemical nociception and inflammatory pain. Fenobam was also found to be without analgesic effect in mGlu5 KO mice, suggesting that its analgesic effect is mediated via mGlu5. Further assessment of both mGlu5 KO mice and the effects of fenobam suggested that mGlu5 may play a role in locomotion, weight gain, and appetite. Fenobam increases locomotive behavior, suggesting that antagonists of mGlu5 may exert analgesic effects without causing dose-limiting sedation. Overall these data suggest that mGlu5 is an important modulator of nociceptive plasticity, and that allosteric modulation of mGlu5 may represent a pharmacologic target for the treatment of pain.

ACKNOWLEDGEMENTS

I have many people to thank. Science is not conducted in a vacuum and the work presented in this thesis was certainly a collaborative effort. I would first like to thank the members of the Gereau Lab and the Washington University Pain Center for their support both professionally and personally throughout the past four years. The Gereau Lab in particular has been a wonderfully energetic place to work and learn. Special thanks go to Mena Morales, Ben Kolber, and Ben Alter who shared the experience of being a Washington University graduate student with me. I am grateful to them for scientific discussions, manuscript and grant readings, and especially friendship. I would like to say a ***big*** thank you to Laura Cavallone, who participated directly in the fenobam experiments described in the third chapter. Laura has been a source of inspiration, a wonderful mentor, and a dear friend. I look forward to continuing our collaboration in the future. Judy Golden has also be an outstanding scientific mentor and a wellspring of knowledge, always available to discuss an obscure reference and to teach a new technique. She also provided me with an in-depth reading of this document and for that I am extremely grateful. I would also like to thank Steve Davidson for simply excellent discussions of pain theory and experimentation.

Beth Conrardy and Lesely Rao played an integral role in several of the fenobam experiments described in Chapter 4. I thank them for this, and for the helpful guidance they provided about navigating the anesthesiology clinical world. Cheng-Shui Zhao provided his expertise in performing the intrathecal DHPG injections. I've been thrilled

to watch Lara Crock, Henry Lai, and Chang Shen Qiu test fenobam and the role of mGlu5 in bladder pain, and look forward to seeing the results from their continued efforts on this project. Chang Shen Qiu and Sherri Vogt deserve special recognition for managing the lab, managing the mouse colony, and just being such kind and generous people. The Division of Comparative Medicine and particularly our mouse care technician Lawrence Fields have also been invaluable in this project. I would also like to thank the younger generation of Gereau Lab personnel, Dan O'Brien, Dani Brenner, Clint Morgan, Patty Stutz, and Joe Demaro Jr. for their enthusiasm and excitement both for conducting experiments and for life in general. I also appreciate the teaching and advice I received from former Gereau lab members, Yarimar Carrasquillo, Santina Chiechio, Magda Zammataro, and Julie Drobish.

I would like to single out my dissertation committee, Tom Baranski, Yu-Qing Cao, Zhou-Feng Chen, Evan Kharasch, and Karen O'Malley for their guidance and help throughout the years. Several members of the Kharasch lab also directly and indirectly contributed to this project. I wish to thank Kristi Stubbert and Andrei Stefanescu for performing the plasma and brain fenobam concentration analysis, and Scott Campbell and Sarah Brown for additional insights and assistance. I would like to thank Room 100, the MSTP office, the DBBS office, and the Anesthesia Research Unit Administrative Office for keeping the wheels behind the scenes well-greased and devoid of hangups. It has been a joy to work with such talented and friendly people as Brian Sullivan, Liz

Bayer, Christy Durbin, Linda Perniciaro, Andrew Richards, Sally Vogt, Diane Smith, Allison Grindon, Amanda Heflin, Lisa Hayes, and Carina Campbell.

Outside of the Washington University community I would like to thank Nitin Agarwal and Rohini Kuner in Germany for providing the SNS-Cre mouse and Jian Xu and Steve Heinemann in San Diego for providing the floxed mGlu5 mouse. I would like to thank Karen Petersen at UCSF for teaching Laura Cavallone, Rob Gereau, and me the heat-capsaicin model. Most of the work presented in this thesis was funded by grants from the National Institutes of Health National Institute of Neurological Disorders and Stroke, specifically to myself as an NRSA (NS064626) and to Rob Gereau as an R01 (NS42595). I would like to thank the NIH for this opportunity. In addition, the Gereau Lab has received funding from a Washington University in St. Louis Neuroscience blueprint core grant that was directly applied to this thesis work.

Graduate and medical school have presented me with their own unique challenges and I would like to thank the many friends I have made through medical school and the MSTP. Without these people the world would be a much darker place. I'm sure I am forgetting someone, but I would like to thank Sam Merrill, Laura Merrill, Brian White, Beth Helmink, Aaron Norris, Jen Griffith, Steve McCartney, Michelle Sabo, Alecia Vogel, Diwakar Turaga, Monica Walker, Nick Foeger, Tom Pearce, and Adam Rouse for sharing adventures great and small, and for their friendship over the years.

My family has played an instrumental role in my education and life. I would especially like to thank my parents for always encouraging me in all that I do, and for

always looking out for my happiness by providing love and support. My sister Lindsey has alway been a supporter of my desire to purpose a career in science and I've been equally impressed by her career as a physical therapist.

The person who has provided me with the most support over the last 5 years is my wonderful and incomparable wife Cindy. She has been my partner not just in medical and graduate school, but in life. She has been there for all the ups and downs of school and other things and without her I'm not sure I would have survived. Thank you Cindy.

Finally I would like to thank my thesis mentor, Rob Gereau. Rob has succeeded in fostering a lab working environment and recruiting people that make being in lab feel very little like a job. Scientific integrity, creativity, enthusiasm, business acumen, a depth of knowledge, and a zest for life are all esteemed qualities even when found individually. When they are encompassed in just one person it is truly something special. I wish to thank Rob for giving me the opportunity to work in the lab and encouraging my personal and professional development. I will always cherish the years I spent at Washington University and in his lab.

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Chapter 1

Introduction to the Role of Metabotropic Glutamate Receptor 5 (mGlu5) in Pain

The study of pain has occupied philosophers, physicians, and scientists throughout recorded history. Pain is a multifaceted sensory and emotional experience that is commonly associated with bodily harm or injury. The English word "pain" has roots in the Latin word *poena* or "punishment." When considering physiological pain, punishment seems appropriate; physiological pain acts as a defense system that allows individuals to avoid or withdraw from noxious and dangerous stimuli. Following tissue damage, tenderness and hypersensitivity at and around the site of injury encourage healing, by punishing an organism for overusing the affected area. Importantly, while physiological pain may be prolonged, it is reversible, and as healing occurs the necessity for pain to serve a protective role diminishes and the pain regresses. However, pain can outlast the injury and become chronic or pathologic.

The existence of chronic pain is a major clinical problem confronting patients and their physicians (National Center for Health Statistics, 2007). This document will focus on the role that metabotropic glutamate receptor 5 (mGlu5) plays in the development and maintenance of chronic pain and the possible clinical utility that mGlu5 antagonists may have in alleviating that pain. In addition I will discuss the role that mGlu5 plays in physiological non-pain mechanisms to provide a context in which to address potential side effects of mGlu5 antagonists.

Pain Terminology and Definitions

Catalogued debate regarding the nature of pain dates to at least ancient Greece where Aristotle viewed pain as an emotion (Perl, 2007). An emotional aspect of pain remains in modern times, and the International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (Loeser and Treede, 2008). While current views of pain are shaped by a detailed understanding of anatomy and neurophysiology, many questions remain. Discoveries over the last several hundred years have shown that specific neuro-anatomical pathways are involved in the transmission of acute painful stimuli from the periphery to higher brain centers, and that these transmissions cross multiple synapses within both the spinal cord and the brain (Milan, 1999). We also know that pain perception can vary greatly depending on environment or context. For example, injury may result in previously non-painful stimuli being perceived as painful. However, the specific mechanisms that underlie these changes are not completely understood. Plasticity at multiple ascending synapses throughout the pain neuraxis likely plays a role in some of the profound changes that can occur in pain perception. In addition modulation of ascending afferents by descending neurons (e.g. pontospinal) within the spinal cord can also affect the perception of painful stimuli (Howorth et al., 2009). Individuals that suffer massive traumatic injuries may report no pain at the time of the injury (Melzack and Wall, 1982). Unfortunately the reverse is also true, and both individuals with only minor injuries (Melzack and Wall, 2003, p. 276), and

those who have no discernible injury (Wolfe et al, 1990, 2010) may report debilitating pain. A classic example is phantom pain, where painful sensations are perceived in a missing limb following amputation. The fact that pain can still be perceived in the missing limb is viewed as evidence that peripheral input is not required to experience pain. In addition, as the peripheral input is absent, changes elsewhere in the nervous system, either in the spinal cord or higher brain regions, must underlie this increased pain perception.

Phantom limb pain is but one example of how plasticity within the nervous system may contribute to sensitization following injury and the development of chronic pain. Nociception is the "neural process of encoding and processing noxious stimuli" (Loeser and Treede, 2008), and the perception of pain relies on the activation of neurons within the peripheral somatosensory system as well as the awareness of that stimulation due to activation of neurons in the spinal cord and brain. Altered pain perception following injury is thought to be due to increased responsiveness and reduced thresholds of neurons in the peripheral and central nervous system. Injuries that result in tissue damage often induce an inflammatory reaction that contributes to the sensitization of peripheral somatosensory neurons, reducing their firing threshold and increasing their responsiveness to stimuli within their receptive fields. This phenomenon is termed peripheral sensitization (Loeser and Treede, 2008). In addition, neurons within the central nervous system may become sensitized and exhibit increased responsiveness to input

from peripheral afferents, a phenomenon known as central sensitization (Loeser and Treede, 2008).

Peripheral and central sensitization occur in nociceptive neurons, which are somatosensory neurons that encode potentially or actually tissue damaging events, known as noxious stimuli (Loeser and Treede, 2008). Peripheral nociceptive neurons have nerve endings in the periphery that transduce and encode noxious stimuli. The perception of pain can arise from direct external stimulation and activation of nociceptors (nociceptive pain) or from damage or disease within the somatosensory system itself (neuropathic pain). It is important to note from the IASP definition of pain above the emotional component — pain is more than just sensation of noxious stimuli, it also has a strikingly negative affective component that can radically alter behavior and quality of life. Unpleasantness, distress, discomfort, and suffering can all be emotional descriptions of pain perception, and an integrated view of pain includes both an emotional and a sensory component. Thus factors that affect both the sensation of pain, such as the enhancement or blockade of peripheral nociceptive neural activity, and the emotional and behavioral responses, such as anxiety, stress, and awareness, may play important roles in pain perception. While acute pain is generally viewed as physiological and protective, prolonged pain that outlasts the inciting stimulus can be detrimental and may be viewed as pathological. Pathophysiological changes within the nervous system can allow pain to be perceived in the absence of ongoing nociception. In addition, the perception of pain can be brought on by a multitude of different inciting stimuli. Pain is a hallmark of many

disease processes and can by classified in multiple ways (Loeser and Treede, 2008; Woolf et al., 1998), including by location (e.g. abdominal pain), by mechanism (e.g. neuropathic pain), as a component of a disease (e.g. arthritis pain), by inciting factor (e.g. post-operative pain), and by responsiveness to medication (e.g. indomethacin responsive headache), just to name a few.

The significant impact pain has on both individuals and society has made it a focus of major research efforts. Much of this research has been conducted in human subjects. However, the ethical and practical limitations of conducting pain research only in humans have resulted in the development of many laboratory animal models for the study of pain. The complex nature of observing and interpreting animal behavior warrants a closer look at some of these models.

The Applicability of Pain Assessment in Rodents to Humans

Much of the evidence and data presented below about the analgesic efficacy of mGlu5 antagonists are derived from behavioral animal models of pain. It is important to discuss some of the limitations of these animal models and how they are thought to apply to human pain conditions. As discussed above, human pain is a subjective experience that can be influenced by a multitude of factors, including level of awareness, surrounding environment, and stress. Animals possess many neuroanatomical pathways for transmitting information regarding peripheral nociception similar to those found in humans (Willis and Westlund, 1997). Additionally, as is true for humans, reported measurements of nociception in mice are susceptible to environmental and experimental state influences such as genetic background (Mogil et al., 1999), housing conditions, experimenter presence, diet, age, gender, (Mogil, 2009) and social cues (Langford, 2006). However, as elegantly stated by Bud Craig "A rat is not a monkey is not a human." (Craig, 2009), and differences between humans and experimental laboratory animals exist with regard to both neuroanatomy, and, importantly, the ability to report painful stimuli. Animals simply cannot verbalize when they are in pain, and surrogate measurements must be used such as withdrawal from stimuli, escape from noxious locations (e.g. extreme heat or cold), licking, shaking, or lifting of injured areas, as well as more complex behavioral readouts such as conditioned place preference (King et al., 2009). Technically we can never *know* whether an animal is in pain and thus hypersensitivity and hyperalgesia (which may include decreased threshold to stimuli and

increased responses to suprathreshold responses), and spontaneous behavior are used as surrogates to infer pain. Of course it should be noted that no purely objective parameters such as specific biomarkers currently exist for pain in humans either, and while human possess the benefit of language for communicating, pain is still a subjective experience in our species as well.

While the inability to determine what experimental animals are subjectively experiencing marks a significant challenge to the direct applicability of animal models to human pain conditions, there are a number of factors that contribute to the utility of animal models of experimental pain, both now and in the foreseeable future. One of the foremost is the ability to use knockout animals to assess the role that specific monogenetic influences play in pain behaviors. Knockout mice have been generated for a number of proteins involved in pain transduction and perception and have greatly contributed to our understanding of the specific roles these proteins play in pain transmission. For example, mice lacking the heat-gated cation channel TRPV1 show deficits in development of heat hypersensitivity following inflammation (Caterina et al., 2000) and mice lacking mu-opioid receptors have profound impairments in their responses to the opioid agonist morphine (Matthes et al, 1996). While important considerations regarding compensation and genetic background exist when assessing genetically modified animals (Larviere et al., 2001), genetically modified mice have proven indispensable in assaying the necessity for a specific protein in pain perception, or the target of action of an analgesic drug. In addition, the testing of animals is beneficial

for economic and ethical reasons : the effects of single genes or pharmacological treatments can be assessed in a battery of pain-related behavioral tests that would be cost-prohibitive (due to sheer numbers) or unethical (e.g. controlled neuropathic pain injury models) to perform in humans.

Specific pain related behavioral tests described in this thesis include the formalin test of spontaneous nocifensive behavior, complete Freund's Adjuvant-induced mechanical and thermal hypersensitivity, and two models of neuropathic pain: the chronic constriction injury model, and the spared nerve ligation model. These models are discussed below.

Spontaneous Formalin-Induced Nocifensive Behavior: The formalin test is a model of acute to moderate duration pain. Spontaneous nocifensive behaviors (including licking, lifting, guarding, and flinching) are induced by the injection of a dilute solution of formalin into the plantar surface of the mouse's hindpaw (Dubuisson ad Dennis, 1977). Formalin concentrations used are generally between 1% and 5% (of a 37% w/w solution of formaldehyde), dissolved in normal saline. Injection volumes for mice are usually limited to 10 microliters, due to volume constraints of the hindpaw. Immediately post injection a characteristic biphasic response is seen, the first phase of which lasts for approximately 5 to 10 minutes, followed by a period of inactivity, and then a second phase of nociceptive behavior beginning around 15 to 20 minutes and lasting approximately another 15 to 20 minutes. Spontaneous nocifensive behaviors cease at

from between 45 to 60 minutes, however hypersensitivity to mechanical and thermal stimuli may remain for days to weeks (Kolber et al, 2010; Wu et al., 2004). Spontaneous nocifensive behaviors can be scored by an observer and the responses of mice in different treatment groups or of different genotypes can be compared.

The first phase of the formalin test is believed to be due to the direct activation of the ion channel TRPA1 (Macpherson et al., 2007; McNamara et al., 2007), although concentrations of formalin that are used for behavioral testing most likely also cause generalized inflammation and no specific tissue damage. Initially it was felt that the second phase was solely due to ongoing inflammation and central sensitization within the spinal cord (McMahon and Koltzenburg, 2005, p.183), however continued discharge of A-delta and C-fibers that begin firing 15 minutes post injection suggest that at least some of the second phase responses are due to ongoing peripheral nociceptor activity (Puig and Sorkin, 1996). Analgesics that are known to modulate human pain, including opioids, NSAIDs, and the anti-convulsant gabapentin (Le Bars et al., 2001; Munro et al., 2007) are known to reduce spontaneous formalin behavior. The formalin test is felt to be one of the most predictive models of acute animal pain, in that drugs that are effective analgesics in humans are also largely effective in reducing acute pain in animals (Le Bars et al., 2001).

CFA-Induced Mechanical and Thermal Hypersensitivity: Complete Freund's Adjuvant (CFA) is an emulsion of heat-killed Mycobacterium in paraffin oil. CFA causes an

inflammatory response when injected into tissues due to its ability to attract macrophages and other immune cells. This results in hypersensitivity to both mechanical and thermal stimuli in animals (Alter et al., 2010; Ren, 1999) and in man (Gould, 2000). Thresholds to mechanical and thermal stimuli can be monitored in resting animals. Baseline measurements are made and then hypersensitivity is induced by subcutaneous injection of 10 microliters of CFA into the dorsal hindpaw. The effects of both drugs and genotype on the development of and recovery from hypersensitivity can be assessed. Mechanical hypersensitivity is commonly assessed on the hindpaw, but can also be assessed on the face or knee joint. Calibrated nylon monofilaments of increasing tensile strengths are applied to the plantar surface of the foot, and the bending force of the filament from which the mouse withdraws is recorded. Sensitivity to heat stimuli is measured in a similar manner, except the mouse is placed on a glass platform and a focused radiant light source is used to deliver a thermal stimuli to the hindpaw. The latency to withdraw from the heat source is used as the behavioral read out (Hargreaves et al., 1988).

Neuropathic Pain Models: The development of neuropathic pain results from direct damage or lesion to the somatosensory nervous system (Loeser and Treede, 2008). Multiple models of neuropathic pain exist derive from different injuries to the nervous system. The most popular models try to mimic the partial nerve injury that is usually seen in neuropathic pain patients by inducing only a partial injury instead of a complete lesion that would only be seen following overwhelmingly severe injuries, such as amputation.

Two of the most common forms of inducing neuropathic pain are to perform a loose ligation around the sciatic nerve using chronic gut suture (Bennett and Xie, 1988) and to selectively lesion two of the three primary distal branches of the sciatic nerve while leaving the third intact (Decosterd and Woolf, 2000). These models are respectively termed the chronic constriction injury model (CCI) and the spared nerve injury (SNI) model. Both models induce hypersensitivity to thermal and mechanical stimuli that is responsive to known analgesics. The SNI model involves the transection of the tibial and common peroneal branches of the sciatic nerve, while leaving the sural nerve intact. This results in hypersensitivity developing on the lateral side of the hindpaw in the territory of the sural nerve, which can be specifically probed for hypersensitivity. The SNI surgery itself is viewed as relatively easy to perform, however testing post injury requires careful attention to the area of the paw being probed as significant differences are found in responsiveness to stimuli applied to different nerve territories (e.g. sural vs. saphenous; Decosterd and Woolf, 2000; Zhao et al., 2004). The CCI model produces swelling and inflammation around the nerve and is intended to damage some but not all of the sciatic axons. It is inherently difficult to reproduce consistently and can result in variable responses to stimuli. However, testing of hypersensitivity is quite simple, because the entire paw of the organism is theoretically hypersensitized. Additional models of neuropathic pain also try to replicate diabetic peripheral neuropathy,drug-induced neuropathic pain, and direct spinal cord injury (Sandkuhler, 2009).

These animal models and others used throughout the pain research field represent powerful tools, especially in the assessment of new pharmacological treatments for painful conditions, while simultaneously allowing for the assessment of potential undesirable effects that might endanger human patients and volunteers. It is also important to note that genetic and pharmacological manipulations of behaving animals may alter the behavioral readouts used to assess the analgesic effect of a given situation. For example, a drug that induces somnolence or causes motor impairment may also alter a behavioral readout surrogate of analgesia that requires intact motor function, independent of its analgesic effect (e.g. paw withdrawal). For this reason in addition to the pain related behavioral tasks presented below I have also assessed locomotion, motor coordination, and other potential effects, which if altered, could confound interpretations of pain-related behavior. Ultimately animal testing is an important and useful component of pain research, so long as the obvious caveats are addressed. Many analgesics currently used in humans have been shown to be efficacious in animal models of pain (Mogil, 2009), and molecules and techniques tested in animals have been successfully applied to the treatment of human pain conditions. Still no panacea for chronic pain exists. While current treatment regimens are very effective in treating some types of pain (e.g. acute post-operative pain), the effective treatment of chronic pain is a largely unmet goal. Also as discussed below, many highly effective analgesics suffer from poor therapeutic index. The need for additional treatments is paramount and the use of animal pain models will be critical to future endeavors.

Examples of Currently Available Pharmacological Therapeutics and Their Limitations

Current treatments for pain rely on a variety of pharmacological therapeutics, surgical interventions, physical therapy, psychological/psychiatric approaches, and their use in combination. Present pharmacological therapies include several different classes of medication such as anti-pyretic agents, nerve-blockers, opioids, and anti-epileptics. While many of these agents are highly effective in controlling pain, they all have specific settings in which they are and are not useful, as well as dose-limiting side effects. Additional drugs in the pharmacological armamentarium may address some of the limitations of currently available therapeutics and bridge current gaps in coverage.

Pain relievers derived from natural products have long been used by human civilizations. Two of the most prominent examples are willow bark preparations that contain salicylic acid (from which the antipyretic analgesic aspirin can be synthesized) and the poppy seed extract opium (which contains the opioid narcotic morphine). Despite centuries of use, the precise mechanisms of action through which these drugs exert their analgesic effects have only come to light in the last half century (Vane, 1971; Pert, 1973). While both salicylate derivatives and opiates are outstanding analgesics, they are not effective in the treatment of all types of pain and have undesirable side effect profiles that can result in significant morbidity when used long term. The discovery of both new analgesics and the analgesic properties of drugs originally developed for different purposes has generated major advances in the ability to treat chronic pain. Here I will

discuss the clinical utility and dose limiting effects of a few classes of analgesic medications.

Non-steroidal Anti-inflammatory Drugs (NSAIDs) and Antipyretics: Arachidonic acid (AA) is synthesized from cell membrane phospholipids in response to a variety of stimuli, including inflammation (Rao and Knaus, 2008). The enzyme cyclooxygenase (COX) catalyzes the conversion of AA to prostaglandin G_2 (PGG_2) and subsequent intracellular reactions result in the production of several additional prostaglandins including the pyretic prostaglandin PGE_2. Several prostaglandins are pro-algesic and can sensitize primary afferent nociceptive neurons (Melzack and Wall, 2003, p. 345; Lopshire and Nicol, 1997) and act synergistically with other inflammatory mediators such as bradykinin (Taiwo and Levine, 1988). The analgesic effects of NSAIDs, including salicylate derivatives such as aspirin, are largely attributed to their ability to act as COX inhibitors and thus decrease the synthesis of prostaglandins (Vane, 1971). NSAIDs have clinical utility in the treatment of pain, especially in conditions associated with inflammation, such as rheumatoid arthritis. However, in rheumatoid arthritis, NSAIDs only provide symptomatic relief and do not alter disease progression (Melzack and Wall, 2003, p. 45). NSAIDs are also useful in the treatment of migraine headache, post-operative pain, and cancer pain (Melzack and Wall, 2003, p. 347). Unfortunately, nonselective COX inhibitors such as aspirin can be toxic to the GI tract and kidney.

Aniline derivatives, such as acetaminophen, are antipyretic and analgesic non-NSAIDs, and have fewer side effects than salicylates when dosing guidelines are maintained (Melzack and Wall, 2003, p. 348). Unfortunately acetaminophen overdose is also the most common cause of acute liver failure (Ostapowicz et al., 2002), which can result in fatality or require liver transplant. In addition, acetaminophen lacks peripheral anti-inflammatory effects, and there are some inflammatory pain conditions, such as rheumatoid arthritis, in which patients prefer NSAIDs over acetaminophen based on effectiveness (Wolfe et al., 2000). Thus NSAIDs and antipyretics represent the classical issues of balancing clinical effectiveness, disease appropriateness, and safety common in other classes of medication.

Opioids: Opioids inhibit the perception of pain by activating endogenous analgesic systems in the spinal cord and brain, which in turn modulate ascending pain transmission along the pain neuraxis (Fields, 2004). Multiple opioid receptor subtypes exist within the nervous system, however, the primary analgesic actions of most opioids in clinical use result from activation of the μ-opioid receptor, which is expressed both pre- and post-synaptically throughout the endogenous pain-modulating circuit. Morphine is the prototypical μ-opioid receptor agonist, and a powerful analgesic (Melzack and Wall, 2003, p. 379). However, concomitant side effects, including nausea, vomiting, narcosis, constipation, respiratory depression, opiate-induced itch, and tolerance (Melzack and Wall, 2003, p. 389-392) require a careful assessment of the risk / benefit ratio when

administering opioids. Additionally, patient perception that addiction is likely (Melzack and Wall, 2003, p. 385-86) may decrease patient enthusiasm for initiating opioid therapy. Regardless of these issues, opioids are a mainstay of cancer and peri-operative pain management. The combination of opioids with adjuvant analgesics (e.g. NSAIDs and anticonvulsants) can also be used to decrease the dose of opioid required to achieve analgesia, and thus decrease the incidence of side-effects. The discovery of new drugs that could act as a opioid adjuvants would be beneficial to the treatment of pain.

Anticonvulsants: Anticonvulsants developed to decrease neuronal firing rates in epilepsy have been found to possess clinical utility in the treatment of neuropathic pain, possibly through the modulation of ion channels required for action potential generation (Tremont-Lukats et al., 2000). Gabapentin is a prime example of the successful use of anticonvulsants as analgesics. Originally developed as an oral anticonvulsant (Bruni et al., 1991; UK Gabapentin Study Group, 1990; US Gabapentin Study Group No. 5, 1993) it has been shown to be effective compared to placebo in the treatment of post-herpetic neuralgia (Rowbotham et al., 1998) and was approved by the FDA for the management of that condition. The precise mechanism of action by which gabapentin exerts its analgesic actions is unknown, however it has been suggested that it mediates some of its analgesic effects via binding to α-2-δ calcium channels (Gee et al., 1998) and inhibiting neurotransmitter release and ectopic discharge (Pan et al., 1999; Taylor et al., 1998). Gabapentin has also been shown to be analgesic in multiple animal pain models (refs).

While generally well tolerated gabapentin administration is not without adverse events, and gabapentin may cause dizziness and somnolence, as well as rare incidents of life-threatening angioedema and suicidal ideation (Pfizer, 2010). Still the use of gabapentin as an analgesic represents a prominent success story in the use of drugs originally developed for other purposes as analgesics.

Future Directions in Analgesia Development: Rational design of entirely new classes of analgesics has been based on recent discoveries in signal transduction of nociceptive stimuli. For example, the algogenic chili pepper extract capsaicin was discovered to induce pain by selectively activating the heat-sensitive cation channel TRPV1 on peripheral nociceptors (Caterina et al., 1997). Unfortunately, some setbacks have occurred, as TRPV1 antagonists have also been shown to induce significant hyperthermia (Gavva et al., 2008). TRPV1 expression in central brain regions, including the hippocampus, and the reported role for TRPV1 in mediating long term depression (LTD) (Gibson et al., 2008), may also represent hurdles to the development of antagonists as analgesics. It is critical to develop new classes of analgesics based on an understanding of their roles in pain processing and plasticity. Glutamate receptor signaling is a prime target for this type of drug development, as its roles in pain processing and plasticity have been extensively characterized during the last decade.

Metabotropic Glutamate Receptor 5 (mGlu5) as a mediator of Peripheral and Central Sensitization and a Potential Therapeutic Target for the Treatment of Pain

Metabotropic Glutamate Receptor Classes and Signaling Cascades: Glutamate is the primary excitatory neurotransmitter in the vertebrate nervous system. It mediates its effects via activation of two main classes of receptors: ligand-gated ion channels known as ionotropic receptors and G-protein coupled metabotropic receptors. Metabotropic glutamate receptors (mGluRs) belong to the Class C family of G-protein coupled receptors (GPCRs) whose structure includes a large venus-flytrap-shaped extracellular N-terminal domain where endogenous ligands, synthetic orthosteric agonists, and competitive antagonists bind. Like all GPCRs, mGluRs possess a seven transmembrane domain region that is responsible for coupling to G-proteins (Bhave et al., 2003). Within the mGluR family eight distinct receptor subtypes have been identified. These receptors are divided into three major groups based on sequence homology, signal transduction mechanisms, and pharmacological profiles (Conn, 2003). In heterologous systems group I mGluRs (mGlu1 and 5) couple to the stimulatory G-protein Gq and subsequently to the activation of phospholipase C (PLC) and the release of intracellular calcium. Group II (mGlu2 and 3) and III (mGlu4, 6, 7, and 8) mGluRs couple to the inhibitory G-protein Gi/o and subsequently inhibit adeylyl cyclase. Group II and III mGluRs also couple to the activation of G-protein coupled inwardly rectifying potassium channels (Knoflach and Kemp, 1998; Watanabe and Nakanishi, 2003) and the inhibition of voltage gated calcium

channels (Conn and Pin, 1997). mGluRs are expressed both pre- and post-synaptically: however group I mGluRs are primarily localized to the postsynaptic density where their activation results in an increase in neuronal excitability, while group II and III mGluRs are primarily localized to presynaptic terminals and function as auto-receptors to regulate neurotransmitter release (Testa et al., 1998; Lovinger and McCool, 1995).

Distribution of mGluRs Throughout the Nervous System: Following the initial discovery of mGluRs in 1991 and the subsequent discovery of a total of eight members of the mGluR family, (Masu et al., 1991), mGluRs have been shown to be expressed throughout the nervous system. Importantly mGluRs are expressed at all levels of the pain neuraxis, including the spinal cord and periphery (Varney and Gereau, 2002). mGluRs are expressed extensively throughout the brain, with the notable exception of mGlu6, which is expressed exclusively in the retina. (Ferraguti and Shigemoto, 2006) mGlu1 exhibits robust expression in the cerebellar cortex, substantia nigra, and hippocampus as well as slightly lower expression in neocortex, amygdala, and striatum (Martin et al., 1992). mGlu5 is found throughout the cerebral cortex, hippocampus, striatum, and amygdala. Of specific interest to pain processing is the expression of mGlu3, 5, and 7 (Varney and Gereau, 2002) within the periaqueductal grey (PAG). Ascending fibers carrying pain and temperature information from the spinal cord via the spinomesencephalic tract synapse within the PAG. In addition, the PAG plays a major role in the descending modulation of pain. Another brain region of particular relevance to pain processing is the amygdala.

Pain is associated with negative emotional responses and in humans the amygdala is an important center for the processing of emotional information. The amygdala is activated during pain in both humans and rodents (Schneider et al., 2001; Ji and Neugebauer, 2008; Kolber, et al., 2010) and activation of group I, II, and III mGluRs within the amygdala (Li and Neugebauer, 2004 & 2006; Kolber et al., 2010) can modulate nociceptive processing. Finally, members of all groups are found in the thalamus, with notable expression of mGlu1, 4, and 7 in nuclei that receive sensory and pain inputs (VPM and/or VPL) (Lorenco Neto et al., 2000).

Expression of all Group I and II mGluRs as well as mGlu4 and 7 has been noted in the spinal cord (Karim et al., 2001; Jia et al. 1999; Chiechio et al., 2002; Varney and Gereau, 2002). Expression levels for mGlu2, 3, and 5 are particularly high in the spinal cord dorsal horn, where first order peripheral sensory neurons carrying nociceptive information synapse onto second order neurons, including ascending projection neurons. In addition, members of all mGluR groups are expressed in the peripheral nervous system within the cell bodies of the dorsal root ganglia in rats (Carlton and Hargett, 2007), and mGlu5 has been identified by immunohistochemistry in human DRGs (Valerio et al., 1997). Over the past two decades a growing body of evidence has contributed to the hypothesis that metabotropic glutamate receptors, and particular mGlu5, are involved in both the induction and maintenance of pain.

The Role of mGlu5 in Pain Plasticity: The first suggestion that mGluRs may play a role in nociceptive transmission came in 1994 from two separate groups. Young et al. (1994) demonstrated that the putatively competitive mGluR antagonist L-AP3 inhibited the excitation of spinal cord dorsal horn neurons induced by the cutaneous application of mustard oil. This work was extended to suggest that the sustained nociception evoked by repeated cutaneous application of mustard oil could also be inhibited by blocking the activation of mGluR mediated intracellular signaling pathways such as PLC (Young et al., 1995). At the same time, Neugebauer et al. demonstrated that L-AP3 reduced the responses of hyperexcitable spinal cord neurons following acute inflammation in the knee joint (Neugebauer et al., 1994). Interestingly, L-AP3 had no effect on either noxious or innocuous pressures applied to the normal knee joint, suggesting that activation of group I mGluRs was selective for inflammation-evoked hypersensitivity and not necessary for the responses elicited under normal conditions. Unfortunately, the non-selective nature of L-AP3 and other early pharmacological tools used to assess the mGluRs (Schoepp et al., 1999) make the interpretation of some of these early experiments difficult. However additional studies using more selective pharmacological agents suggest that activation of group I mGluRs in both the periphery and the spinal cord is indeed pronociceptive (Neugebauer et al., 1999; Dolan and Nolan, 2000; Bhave et al., 2001; Karim et al., 2001).

A principal mechanism by which mGlu5 promotes hypersensitivity is through the modulation of nociceptive transduction channels in peripheral terminals of primary afferent neurons. For example, peripheral injection of the group I mGluR agonist

R,S-3,5-dihydroxyphenylglycine (DHPG) results in thermal hypersensitivity in mice (Bhave et al., 2001; Hu et al, 2002). This is believed to result from the activation of a complicated signaling pathway where group I mGluR activation couples to PLC activation, and results in the generation of diacylglcerol, which is metabolized into the inflammatory mediator arachidonic acid. Further metabolism of arachidonic acid results in the production of prostaglandins via the cyclooxygenase (COX) pathway and the eventual sensitization of the capsaicin receptor TRPV1 (Hu et al., 2002), which is required for the molecular changes underlying inflammation-evoked thermal hypersensitivity (Caterina et al., 2000; Davis et al., 2000). In sensory neurons, DHPG-induced enhancement of calcium responses following application of the TRPV1 agonist capsaicin has been primarily attributed to mGlu5 (Hu et al., 2002). mGlu5-mediated potentiation of these capsaicin-evoked responses is attenuated by both the PLC inhibitor U73122 and the DAG-lipase inhibitor RHC-8026719, suggesting a prominent role for mGlu5- stimulated, PLC-activated PIP_2 signaling pathways in thermal hyperalgesia. Additionally, the potentiation of these capsaicin-evoked responses is also blocked by the COX inhibitors indomethacin and ibuprofen, and the prostanoid receptor antagonist SC-51089. Results from *in vivo* studies demonstrate that inhibitors of mGlu5 (MPEP) and COX (indomethacin, aspirin) block DHPG-induced thermal hyperalgesia (Hu et al., 2002). Taken together, these data suggest that mGlu5-stimulated PLC activation in sensory neurons – and the subsequent activation of downstream prostaglandin inflammatory mediators – promotes thermal hyperalgesia via modulation of TRPV1.

A prominent role for mGlu5 in the development of central sensitization has also been suggested. Modulation of ion channels within the spinal cord may contribute some of the molecular changes that underlie central sensitization (Ji et al., 2003). For example, decreased potassium channel function or effectiveness could manifest in increased neuronal excitability. A specific ion channel that is expressed in dorsal horn neurons and is known to modulate neuronal excitability is $K_V4.2$, which contributes to the fast transient outward potassium current (I_A) (Hu et al., 2003). Genetic deletion of $K_V4.2$ results in increased excitability of dorsal horn neurons and increased sensitivity at baseline to mechanical stimuli. Interestingly however, $K_V4.2$ knockout mice do not demonstrate increased hypersensitivity following activation of extracellular signal-regulated kinase (ERK) (Hu et al., 2006), a known modulator of pain hypersensitivity (Ji et al., 1999). ERK is an integrator of intracellular signaling cascades that are initiated in the spinal cord following painful stimuli, and ERK activation contributes to central sensitization (Ji et al., 2009). The activation of mGlu5 in the dorsal horn of the spinal cord functionally couples to the activation of the ERK (Karim et al., 2001) and the subsequent phosphorylation of Kv4.2 (Hu et al., 2007), which results in increased excitability of dorsal horn neurons. This mGluR5-ERK-$K_v4.2$ regulation of dorsal horn neuronal excitability is believed to represent a cellular underpinning of inflammation-induced central sensitization in the spinal cord. Thus, both peripheral and central activation of mGlu5 has been demonstrated to modulate pain plasticity.

Allosteric Modulation of mGlu5

Exciting advances in recent years have yielded numerous small molecule allosteric modulators of mGluRs that bind within the transmembrane domain at a topographically distinct location from the glutamate binding site. Pharmacological dissection that provides much of the evidence of the role mGlu5 plays in pain plasticity has been largely conducted using these drugs. Allosteric modulators mediate their effects by exhibiting one or more of three pharmacological properties. First, allosteric modulators can exhibit affinity modulation and alter the affinity of the receptor for its endogenous ligand. Second, efficacy modulation may occur such that the binding of an allosteric modulator alters the strength of the downstream signaling cascades induced by the orthosteric ligand. Finally some allosteric modulators may have positive or negative intrinsic activity on the receptor itself such that they function as agonists or inverse agonists regardless of the binding of the orthosteric ligand. With respect to mGlu5, positive allosteric modulators (PAMs) increase, and negative allosteric modulators (NAMs) decrease, the response of the receptor to glutamate. (Conn et al., 2009)

The ligand binding site often has high sequence homology across members of a family of GPCRs, making development of selective agonists or antagonists against a specific receptor subtype difficult. However, modern high-throughput screening for receptor activity modifiers allows for the identification of compounds that bind elsewhere in the receptor, and has allowed for the development of allosteric modulators that have improved receptor subtype specificity when compared to compounds that bind at the

endogenous ligand binding site (Conn et al., 2009). Additionally, some allosteric modulators do not possess intrinsic activity at the receptor, and only exert an effect when an orthosteric ligand is bound. These molecules would theoretically exhibit activity-dependence, only modulating the system when and where it is activated under physiological conditions. For these reasons, allosteric modulators represent attractive candidates for development as pharmacological agents that target mGluRs.

The development of the non-competitive mGlu5 antagonist MPEP (Gasparini et al., 1999) addressed a need for a selective pharmacological tool to probe the role of mGlu5 in chronic pain. While the related compound MTEP (Cosford et al., 2003) is generally thought to be more potent and selective (Lea and Faden, 2006) much of the mGlu5 research over the past decade has utilized MPEP. Systemic MPEP administration reverses mechanical hyperalgesia induced by the inflammatory agents Complete Freund's Adjuvant (CFA) and carrageenan, and oral administration was found to have improved gastrointestinal safety over the NSAIDs indomethacin and diclofenac (Walker et al., 2001a). In addition peripherally administered MPEP was found to significantly reduce CFA induced inflammatory hyperalgesia (Walker et al., 2001b) suggesting a role for peripherally expressed mGlu5 in pain. Bhave et al. (2001) further expanded upon this work by demonstrating the expression of group I mGluRs on the peripheral terminals of nociceptors, and showing that peripheral injection of the group I mGluR agonist DHPG induces thermal hypersensitivity in mice. This DHPG induced hypersensitivity was blocked by peripheral injections of MPEP. Furthermore, peripheral injections of MPEP

were able to prevent and reduce spontaneous behavior following intraplantar formalin injection (Bhave et al., 2001).

Neuropathic pain has also been suggested to be susceptible to modulation by mGlu5 antagonists. Intrathecal injection of the mGlu5 antagonist SIB-1757 is able to reduce mechanical allodynia following spinal nerve ligation (SNL) as well as reverse SNL-induced thermal hyperalgesia (Dogrul et al., 2000). In addition, intracerebroventricular (i.c.v.) injections of MPEP dose-dependently inhibit cold hypersensitivity following chronic constriction injury (CCI) of the sciatic nerve in rats (Urban et al., 2003). Brain receptor occupancy studies following systemic MPEP injections suggested that >90% receptor occupancy is required for effective analgesia. Of note, neither intrathecal nor intraplantar injections of MPEP were found to be effective in reducing cold hypersensitivity post-CCI (Urban et al, 2003), however in both cases only one dose of MPEP was tested and no dose-response measurements were performed. Furthermore, a separate study found that systemic MPEP injections were able to reduce mechanical hypersensitivity induced by multiple neuropathic pain models (Zhu et al., 2004), indicating that multiple sensory modalities may be susceptible to modulation by mGlu5 antagonists following neuropathic injury.

Taken as a whole, the literature suggests that MPEP is analgesic in multiple nociceptive modalities, and may exert its effects at multiple sites within the pain neuraxis (**Table 1**). This would seem to provide strong evidence that mGlu5 plays a prominent role in pain plasticity. However, suggestions that MPEP may act both as a weak NMDA

receptor antagonist with micromolar potency (O'Leary et al., 2000; Movsesyan et al., 2001; Lea and Faden, 2006), and as an inhibitor of the norepinephrine transporter (Heidbreder et al., 2003), have tempered the enthusiasm that it is functioning exclusively via mGlu5. The continued quest for highly selective mGlu5 antagonists was greatly furthered in 2005 by researchers at Hoffman-LaRoche in Switzerland (Porter et al., 2005). They demonstrated that the non-benzodiazepine anxiolytic fenobam is a potent and selective negative allosteric modulator of mGlu5 with a chemical structure distinct from MPEP. This finding was especially exciting as fenobam had previously been tested in clinical trials in the 1980s where it was found to be an effective anxiolytic with a good safety profile (Pecknold et al., 1980, 1982; Lapierre and Oyewumi, 1982). However, due to potential side effects and (during the 80s) an unknown mechanism of action, fenobam was never pursued as a commercial product.

Interestingly, in addition to its analgesic properties, the mGlu5 antagonist MPEP has also been demonstrated to have anxiolytic effects (Varty et al., 2005; Ballard et al., 2005). Furthermore MPEP (Yan et al., 2005), and mGlu5 antagonists in general (Dolen et al., 2007), have been suggested as potential therapeutics for the treatment of Fragile X syndrome (FXS). The hypothesis that fenobam would function as a clinical therapeutic for FXS in humans was recently tested (Berry-Kravis et al., 2009). Fenobam demonstrated some mild beneficial effects and was found to be without significant adverse effects in both healthy volunteers and FXS patients . Prior to the work presented in this thesis, the efficacy of fenobam in the treatment of pain was untested.

Table 1: The Effects of mGlu5 Pharmacological Manipulation on Animal Behavioral Models of Pain

BEHAVIORAL MEASURE	NOXIOUS AGENT	ANTAGONIST	SPECIES	RESULT	REFERENCE
Spontaneous Nocifensive Behavior	DHPG (intrathecal)	-	Mouse	DHPG Elicits Nocifensive Behavior	Karim (2001)
	DHPG (intrathecal)	Intrathecal MPEP	Mouse	MPEP Reduces DHPG Induced Nocifensive Behavior	Karim (2001)
	Formalin (intraplantar)	Systemic MPEP	Rat	MPEP Reduces Spontaneous Formalin Behavior	Zhao (2004)[a]
	Formalin (intraplantar)	Intrathecal MPEP	Mouse	MPEP Reduces Spontaneous Formalin Behavior	Karim (2001)
	Formalin (intraplantar)	Intraplantar MPEP	Mouse	MPEP Reduces Spontaneous Formalin Behavior	Bhave (2001)[b]
Heat Hypersensitivity	DHPG (intraplantar)	-	Mouse	DHPG Decreases Thermal Withdrawal Latency	Bhave (2001)
	DHPG (intraplantar)	Intraplantar MPEP	Mouse	MPEP Prevents Reduced Thermal Withdrawal Latency	Bhave (2001)
	CFA (intraplantar)	Systemic MPEP	Rat	MPEP Reduces Thermal Hypersensitivity	Zhao (2004)
Mechanical Hypersensitivity	DHPG (intraplantar)	-	Rat	DHPG Decreases Mechanical Withdrawal Thresholds	Walker (2001b)
	DHPG (intrathecal)	-	Sheep	DHPG Decreases Mechanical Withdrawal Thresholds	Dolan (2001)[c]
	CFA (intraplantar)	Systemic MPEP	Rat	MPEP Reduces CFA-Induced Mechanical Hypersensitivity	Zhao (2004)
	CFA (intraplantar)	Oral MPEP	Rat	MPEP Reduces CFA-Induced Mechanical Hypersensitivity	Walker (2001a)[d]
	Neuropathic Injury (CCI)	Systemic MPEP	Rat	MPEP Reduces Mechanical Hypersensitivity	Zhao (2004)
Cold Hypersensitivity	Neuropathic Injury (CCI)	Systemic MPEP	Rat	MPEP Reduces the Number of Withdrawals on a Cold Plate	Urban (2003)[e]

a) MPEP also results in motor impairment; b) this paper also demonstrated immuno-electronmicroscopy of mGlu5 on unmyelinated nerve terminals in the skin; c) 50 nmol DHPG induced hypersensitivity, however higher doses were without effect; d) oral MPEP did not impair motor coordination; e) intraplantar and intrathecal MPEP were without effect; Systemic MPEP is administered i.p.; CFA - complete Freund's Adjuvant; CCI - Chronic Constriction Injury

Summary of Findings: mGlu5 as a Target for the Treatment of Pain

Both peripheral and central activation of mGlu5 has been suggested to modulate pain plasticity, and pharmacological antagonism of mGlu5 at peripheral and central sites has been suggested to be analgesic. It is therefore conceivable that pharmacological therapeutics designed to modulate mGlu5 would represent viable analgesics in humans. However, as discussed above, the questionable selectivity of MPEP has prevented the unambiguous determination of the role that mGlu5 plays in pain.

Direct testing of the necessity of mGlu5 in pain plasticity using genetically modified animals has not been previously conducted. One of the primary goals of this thesis was to assess the pain related behaviors of a colony of mGlu5 KO mice as compared to their WT littermates. This was intended to test directly the assumption that mGlu5 is required for the full expression of pain-related behaviors, while simultaneously avoiding the issue of antagonist selectivity. In Chapter 2 I present evidence that mGlu5 KO mice have deficits in several nociceptive behaviors, suggesting that mGlu5 is required for the full expression of pain.

All previous work involving the study of mGlu5 in pain has been performed using animal models and other pre-clinical experiments. Future endeavors aimed at the development of mGlu5 antagonists as analgesics would be aided by an antagonist that has been shown to be clinically viable and safe in human subjects. Therefore, one of the goals of this thesis was to test the hypothesis that fenobam is analgesic in rodents, thus providing the necessary preclinical evidence to proceed with future clinical trials in

humans. Results from studies suggesting that fenobam is analgesic in rodents are presented in Chapter 3. Additionally, the demonstration that mGlu5 antagonists are devoid of analgesic efficacy in mGlu5 KO mice would provide compelling evidence that they are mediating their analgesic effects by inhibiting mGlu5. Therefore another goal of this thesis was to assess the analgesic effects of both fenobam and MPEP in mGlu5 KO mice. Results from these experiments are also presented in Chapter 3.

Finally, future clinical development of mGlu5 antagonists as analgesics will not only depend on analgesic efficacy, but also a lack of debilitating or dose-limiting side effects. An assessment of the locomotor and motor-coordination related behaviors of mGlu5 KO mice as well as the effects of fenobam on these behaviors is presented in Chapter 4. While mGlu5 KO mice seem to have altered locomotor behavior that is also induced by fenobam in WT animals, it is possible that these effects may not impede the development of analgesics as mGlu5 antagonists. One potential way to avoid centrally-mediated side effects would be to use peripherally restricted mGlu5 antagonists. As mentioned above, mGlu5 is expressed throughout the pain neuraxis and both peripheral and central receptors have been proposed to play a role in pain-related behaviors. Thus peripheral receptors could theoretically be selectively or preferentially targeted over central receptors to induce analgesia without inducing centrally mediated side effects. Unfortunately neither the precise role of peripheral mGlu5 in pain, nor the existence of any peripherally restricted mGlu5 antagonists needed to test this hypothesis, are presently known. In order to assess the necessity of peripheral mGlu5 in pain I sought to test the

pain related behaviors of a colony of mice in which mGlu5 has been selectively deleted only from peripheral nociceptors. In Chapter 5 I present findings that suggest that peripherally restricted mGlu5 KO mice do not have alterations in their pain related behaviors. While this decreases enthusiasm for the development of peripherally restricted mGlu5 antagonists as analgesics, taken together the bulk of the data presented in this thesis suggest that antagonism of mGlu5 may represent a viable therapeutic option for the treatment of pain in humans.

Chapter 2

The Pain Related Behaviors of mGlu5 KO Mice

INTRODUCTION

As discussed above, mGluRs modulate neurotransmission by coupling to intracellular signaling cascades (Niswender and Conn, 2010). In addition to playing important roles in synaptic plasticity, learning and memory, anxiety, and other neurological disorders, mGlu5 is expressed at synapses throughout the pain neuraxis (Varney and Gereau, 2002) where it has been shown to play a role in the modulation of multiple nociceptive modalities. mGlu5 agonists induce hypersensitivity when administered both peripherally and centrally (Bhave et al., 2001, Kolber et al., 2010), and intrathecal injections cause spontaneous nociceptive behaviors in mice (Karim et al., 2001). Conversely mGlu5 antagonists have analgesic effects when delivered systemically, (Zhu et al., 2004; Walker et al., 2001a) intrathecally, (Karim et al., 2001), and peripherally (Bhave et al., 2001; Walker et al., 2001b; Zhu et al., 2005). However, there is some controversy regarding the selectivity of these antagonists, and suggestions have been made that their analgesic effects are not mediated entirely by mGlu5 (O'Leary et al., 2000; Movsesyan et al., 2001; Lea and Faden, 2006). The issue of antagonist selectivity will be directly addressed in chapter 3. Here I sought to circumvent the issue of antagonist selectivity by using a genetic strategy to test the role of mGlu5 in pain. In this section I present data from mouse pain models used to assess the nociceptive behaviors of mGlu5 KO mice (Jia et al., 1998) and their WT littermates. These mice were generated in the lab of John Roder at the Samuel Lunenfeld Research Institute in Toronto, Ontario. A 0.4-kb fragment containing a portion of exon 1 and intron 1 was deleted and replaced with a 1.8-kb fragment containing a PGK-neomycin cassette.

I used a battery of inflammatory and neuropathic pain models, including DHPG- and Complete Freund's Adjuvant (CFA)-induced hypersensitivity, spontaneous formalin behavior, and both chronic constriction injury- and spared nerve injury-induced hypersensitivity. I have found that mGlu5 deficient mice have reduced responses in multiple pain behavioral models.

METHODS

Animals: Experiments were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Animal Care and Use Committee of Washington University School of Medicine. mGlu5 KO mice (6 to 8 weeks old) were bred inhouse on a C57BL/6 background and compared to WT littermates (Jia et al., 1998). Blinding to genotype was accomplished by using coded ear tag identification numbers and only breaking the code at the end of the experiment. Unless otherwise specified only male mice were tested. All mice were group housed on a 12/12-light/dark schedule with lights on at 0600 CST and *ad libitum* access to food and water.

PCR Genotyping: Genotyping of mice bred inhouse was performed using standard PCR techniques with primers targeted to exon 1 of the mGlu5 locus. Three primers were used, a forward oriented common primer upstream of the deleted segment, and two reverse primers — a WT primer within the deleted segment, and a KO primer within the neomycin cassette.

Primers:

1) Common Primer — oIMR1034 — CAC ATG CCA GGT GAC ATC AT

2) Wild Type Primer — oIMR1035 — CCA TGC TGG TTG CAG AGT AA

3) Knock Out Primer — oIMR2060 — CAC GAG ACT AGT GAG ACG TG

Genotyping Reaction Master Mix (per reaction):

1) MilliQ Water: 8.4 µl

2) GoTaqFlexi (Promega) 5x Buffer: 4 µl

3) $MgCl_2$ (25 mM): 2 µl

4) WT Primer: 1 µl

5) Common Primer: 2 µl

6) KO Primer: 1 µl

7) dNTPs (10 mM): 0.4 µl

8) GoTaq (Promega): 0.2 µl

9) Extracted Tail DNA: 1 µl

PCR Protocol:

Cycle 1 (1x): Step 1: 94°C for 3 minutes

Cycle 2 (35x): Step 1: 94°C for 30 seconds

Step 2: 62°C for 45 seconds

Step 3: 72°C for 45 seconds

Cycle 3 (1x): Step 1: 72°C for 2 minutes

Hold at 4°C until agarose gel separation

Gel Separation: PCR product separation on a 1.5% agarose gel yields 2 bands, a KO band at ~650 bp, and a WT band at ~440 bp. Heterozygous animals have both bands.

Immunohistochemistry: WT and mGlu5 KO littermate mice were deeply anesthetized with a 100 µl injection of ketamine, acepromizine, and xylazine (KAX) (42.86 mg/ml, 8.57 mg/ml, and 1.43 mg/ml KAX respectively), then perfused transcardially with 0.5% procaine in PBS (37^{o}C), followed by 20 ml of ice-cold 4% paraformaldehyde dissolved in PBS. The brain and spinal cord were dissected immediately and cryoprotected overnight in 30% sucrose dissolved in PBS. Tissue samples were mounted in Sakura Tissue-Tek O.C.T. Compound (VWR) and frozen on dry-ice and then stored at -80^{o}C until sectioning. Coronal sections (30 µm) were made on a cryostat and refrigerated in PBS (4^{o}C) until immunostaining. All steps described below were carried out while gently rocking on an orbital shaker. All steps were carried out at room temp except for primary incubation. Sections were washed three times for 5 minutes each in 0.1 M PB, incubated in 0.3% H_2O_2 and 10% methanol in 0.1 M PB for 30 min, then washed again in 0.1 M PB (3 times, 5 min each). Sections were blocked for 2 hours in normal goat serum and 0.3% Triton-X (Sigma-Aldrich, St. Louis, MO), then incubated overnight at 4^{o}C in rabbit anti-mGlu5 (1:3K, Millipore, Cat. # 06-451) dissolved in blocking solution. The next day sections were washed three times for 10 minutes each in 0.1 M PB, and incubated in goat anti-rabbit conjugated to Alexa Fluor 488 (1:1K, Invitrogen). Finally sections were rinsed again in 0.1 PB (3 times, 10 min each), wet mounted, and imaged on an Olympus BX51 compound fluorescent microscope. Exposure times used for WT and KO tissue samples were identical and are reported in the results.

Reagents and Drugs used in Behavioral Experiments: (RS)-3,5-dihydroxyphenylglycine (DHPG) was purchased from Tocris (Ellisville, MO) and dissolved in 0.9% normal saline on the day of the experiment. Complete Freund's Adjuvant (CFA), formalin, and acetone were purchased from Sigma-Aldrich (St. Louis, MO). Formalin was diluted in 0.9% normal saline on the day of the experiment.

General behavioral testing conditions: All behavioral tests were conducted by an observer blind to genotype, in a room that was isolated from other activity, and in the presence of white noise. Room temperature was approximately 25^{o}C. Mice were acclimated to the testing environment starting 2 to 3 hours after the beginning of the light cycle. Acclimation was always performed for >2 hours prior to any behavioral testing. All paw injections were subcutaneous, and were made into the plantar surface of the right paw.

Intraplantar DHPG-Induced Heat Hypersensitivity: Heat thresholds were measured using a modified Hargreaves' test (Hargeaves et al., 1988). Mice were placed in individual transparent Plexiglas boxes (10L x 10W x 15H cm) on an elevated glass platform. A continuous radiant heat source was delivered through the glass onto the surface of each hind paw (IITC Life Sciences, Woodland Hills, CA), and the latency for animals to withdraw their paw from the glass surface was measured. The idle intensity of the heat source was set to 2% and the active intensity was set to 15% with a 20-second

cutoff. Baseline measurements were collected one day before collecting hypersensitivity measurements and animals were habituated to the testing environment separately on each day. For measurement of baseline thresholds 5 individual measurements were made per paw and averaged. Hypersensitivity was created by right hindpaw subcutaneous injection of 50 nmols of DHPG dissolved in normal saline. The injection volume was 10 µl of 5mM DHPG solution. Withdrawal latencies were measured at 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, and 240 minutes. Every two measurements were averaged to create one time point (e.g. 15 min. and 30 min. were averaged as the 30 min. time point, etc.).

Intrathecal DHPG-Induced Spontaneous Behavior: Mice were placed in individual transparent Plexiglas boxes (35L x 25W x 25H cm) on an elevated glass platform. DHPG (50 nmols dissolved in normal saline) was injected intrathecally into the lumbar spinal cord at the level of the iliac crest using a Hamilton syringe and a 30 gauge needle. The injection volume was 5 µl of a 100 mM DHPG solution. The total time spent in spontaneous nocifensive behavior, defined as caudally oriented licking of the flanks, hindpaws, or tail, was scored in 5 minute intervals for 15 minutes following DHPG injection.

Spontaneous Formalin-Induced Nocifensive Behavior: Mice were placed in transparent Plexiglas boxes (10L x 10W x 15H cm) on a glass surface and 10 µl of 2% dilute formalin solution (Sigma-Aldrich, St. Louis, MO) was injected subcutaneously into

the plantar surface of the right hind paw. The time spent in nocifensive behavior, defined as licking, lifting, or flicking of the injected paw, was scored in five-minute intervals for 60 minutes after paw injection.

Complete Freund's Adjuvant (CFA)-Induced Heat Hypersensitivity: Heat thresholds were measured using a modified Hargreave's apparatus in the same manner as described for DHPG-induced heat hypersensitivity. Baseline measurements were collected one day before collecting hypersensitivity measurements and animals were habituated to the testing environment separately on each day. For measurement of baseline thresholds 5 individual measurements were made per paw and averaged. CFA-induced hypersensitivity was created by subcutaneous injection of 10 µl of CFA (Sigma-Aldrich, St. Louis, MO; 1 mg/ml) into the right paw. Withdrawal latencies were measured at 30, 60, 90, 120, 150, and 180 minutes, and 1, 2, 4, and 7 days. For time points minutes after injection individual measurements were made. For time points days after injection, 5 independent measurements were averaged.

Measurement of CFA-Induced Mechanical Thresholds: Mechanical sensitivity was measured using nylon von Frey filaments (North Coast Medical, San Jose, CA). Mice were habituated in individual transparent Plexiglas boxes (10L x 10W x 15H cm) on an elevated wire mesh surface. Starting with the smallest filament (0.008 g), successively larger filaments were applied to the mouse's hindpaw between the anterior and posterior foot pads. The smallest filament that evoked responses in 3 out of 5 applications was

taken as the mechanical threshold for each trial. Baseline measurements were collected one day before collecting hypersensitivity measurements and animals were habituated to the testing environment separately on each day. For measurement of baseline thresholds 3 individual measurements were made per paw and averaged. For CFA-induced hypersensitivity 10 µl of CFA (Sigma-Aldrich, St. Louis, MO; 1 mg/ml) was injected into the right paw and withdrawal latencies were measured at 1, 2, and 3 hours, and 1, 3, 5, and 7 days. For time points hours after injection individual measurements were made. For time points days after injection, 3 independent measurements were averaged.

Chronic Constriction Injury Model: Mice were placed in individual transparent Plexiglas boxes (10L x 10W x 15H cm) on a wire mesh and spontaneous nocifensive behavior following the application of one drop of acetone applied to each hindpaw using a 1 ml syringe was measured for one minute post application. Two measurements were taken per paw and averaged with at least 5 minutes between applications. Spontaneous nocifensive behavior was defined as licking, shaking, or lifting of the hindpaw to which acetone was applied. Hypersensitivity to acteone was induced via chronic constriction injury of the sciatic nerve using a modified version of the model developed by Bennett (Bennett & Xie, 1988) Briefly, one day after baseline measurements mice were anesthetized with a combination of ketamine, acepromizine, and xylazine (KAX) (114mg/kg, 1.14 mg/kg, and 5.76 mg/kg KAX respectively), a small incision in the left thigh was made, and the sciatic nerve was exposed. Two loose ligatures of 6-0 chromic gut suture were tied around the sciatic nerve one millimeter apart. The wound was

irrigated with sterile saline and closed with 6-0 silk suture. Animals were allowed to recover for 3 days, then spontaneous nocifensive behavior to acetone was determined as described above. Acetone-induced nocifensive behavior was measured 3, 5, 7, 10, 14, 21, and 28 days post injury.

Spared Nerve Injury Model: Mechanical hypersensitivity following spared nerve injury was performed as described previously (Alter et al., 2010). Briefly, mice were placed in individual transparent Plexiglas boxes (10L x 10W x 15H cm) on a wire mesh and baseline withdrawal latencies to Von Frey filaments were obtained from the sural nerve territory (lateral surface of the hindpaw) using the up-down method (Chaplan et al., 1994). One day after baseline measurements mice were anesthetized with pentobarbital, a small incision in the thigh was made, and the three main branches of the sciatic nerve were exposed. The common peroneal and tibial nerves were ligated with 8-0 silk suture and cut distal to ligation. One millimeter of distal nerve stump was removed, and the surgical wound was closed with 7 mm surgical clips. After 3 d of recovery, clips were removed. Withdrawal latency to mechanical thresholds in the sural nerve territory was obtained 3, 5, 7, 10, 14, 21, and 28 days post injury.

Data Analysis: Statistical analysis of behavioral data was performed using Prism 5.0 (GraphPad Software, Inc.).All data collected over multiple time points from the spontaneous formalin and DHPG, mechanical and thermal hypersensitivity, and

neuropathic pain models, were statistically analyzed using a Bonferroni multiple comparison test after a two-way ANOVA. Summed data from these tests were analyzed using a two-tailed t-test when comparisons were made between two groups or a Bonferroni multiple comparisons tests after a one-way ANOVA when comparisons were made between more than two groups. Comparisons made between 2 groups at one time point (e.g. baseline thermal withdrawal latencies) were made using a two-tailed t-test. In all studies, the accepted level of significance was $p < 0.05$. In all figures data are reported as mean +/- SEM. Comparisons between baseline and later data points were done as a one-way ANOVA with a Dunnett's Multiple Comparison Post Test.

RESULTS

Verification of mGlu5 Deletion

PCR Genotyping: PCR yielded bands of expected size that were readily separable via agarose gel electrophoresis (data not shown).

Immunohistochemsistry: IHC of brain and spinal cord slices from WT animal identified by PCR demonstrated robust mGlu5 immunoreactivity in the CA1 region of the hippocampus, the dentate gyrus, the striatum, and the dorsal horn of the spinal cord. In agreement with previous reports (Jia et al., 1998), this immunoreactivity was absent in animals identified as KOs (**Figure 1**).

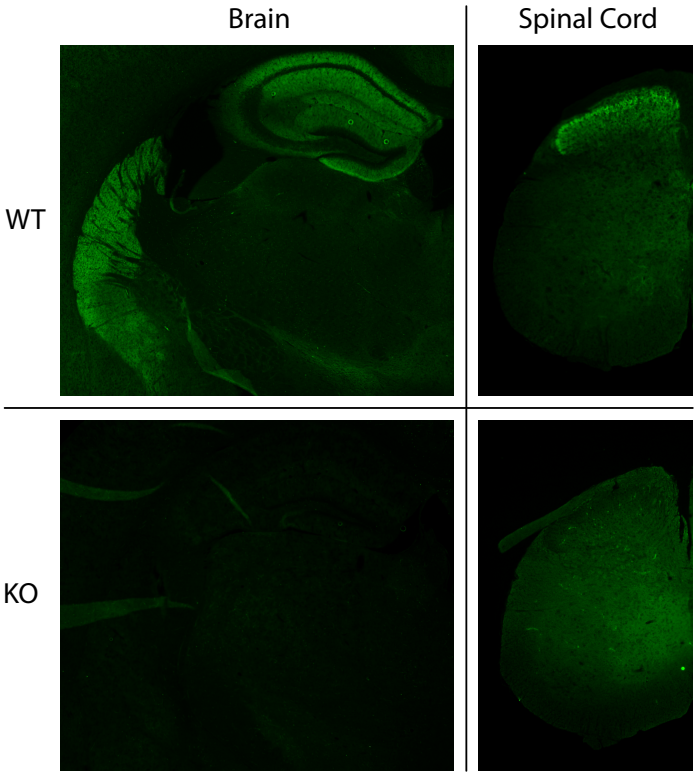


Figure 1: Lack of mGlu5 Protein Expression in mGlu5 KO Mice.

Anti-mGlu5 immunostaining demonstrating a lack of mGlu5 in KO brain and spinal cord. Anti-mGlu5 (1:3K) primary antibody and Alexa 488 (1:1K) secondary. Brain is imaged using a 4x objective and a ⅓ second exposure. Spinal cord is imaged using a 4x objective and a ½ second exposure.

Baseline Sensitivity of mGlu5 KO Mice to Mechanical and Thermal Stimuli: Baseline sensitivities to heat as measured using the Hargreaves apparatus (**Figure 2a**), evaporative cooling as measured by application of acetone (**Figure 2b**), and mechanical sensitivity (**Figure 2c**) as measured using nylon Von Frey filaments, were not different between WT and mGlu5 KO mice.

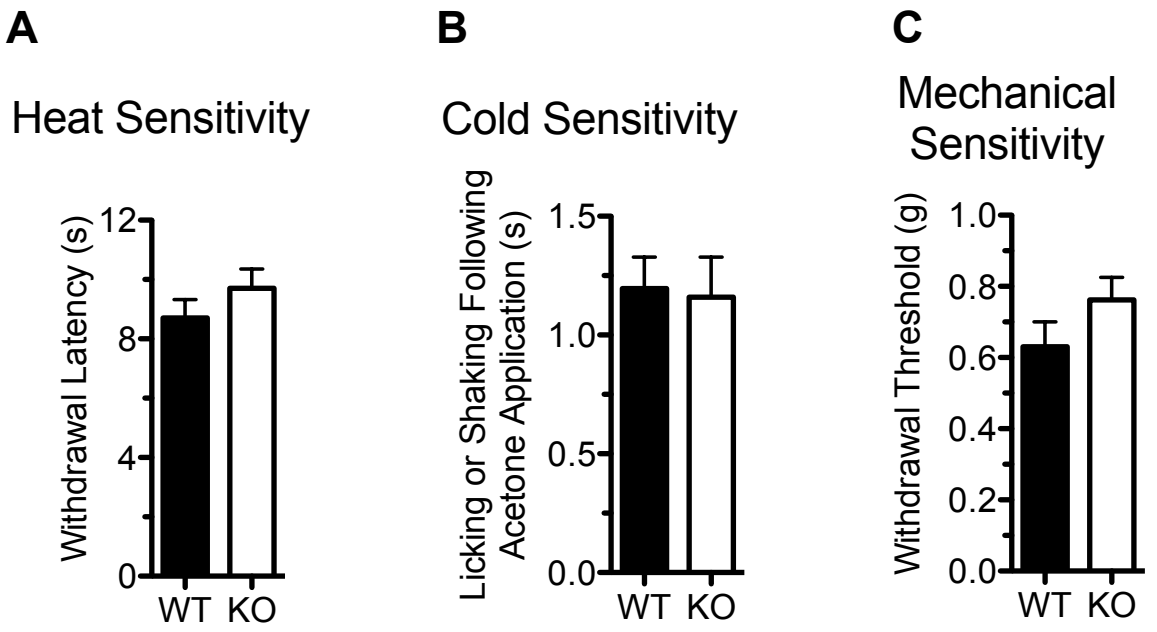


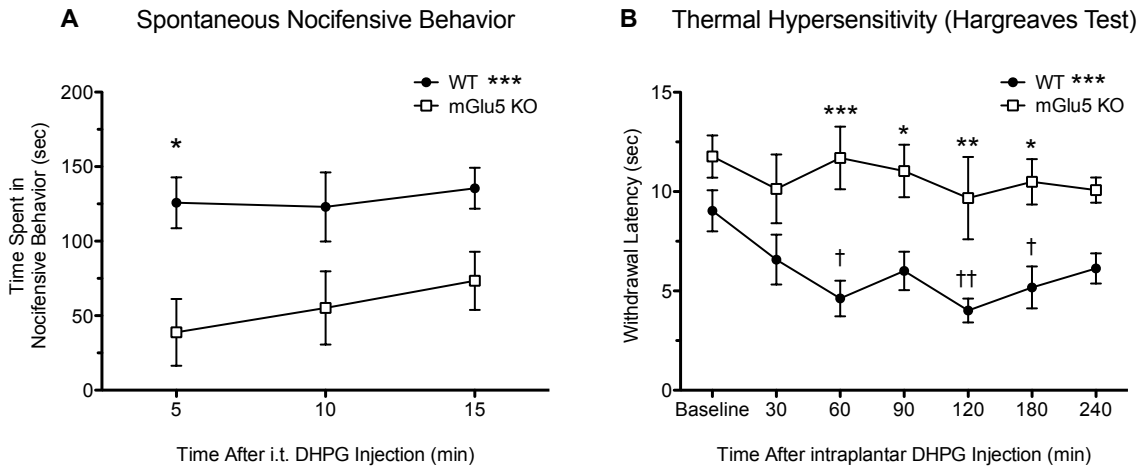
Figure 2: Baseline Thermal and Mechanical Sensitivity are Not Altered in mGlu5 KO Mice

A) Heat Sensitivity(WT = 8.7 +/- 0.6 sec; KO = 9.7 +/- 0.6 sec; n = 30 per group), B) cold sensitivity (WT = 1.2 +/- 0.13 sec; KO = 1.2 +/- 0.17 sec; n = 26 per group for WT, 20 per group for KO), and C) mechanical sensitivity (WT = 0.63 +/- 0.07 g; KO = 0.76 +/- 0.06 g; n = 20 per group) were not altered by a lack of mGlu5. Data are presented as averages of both paws +/- SEM.

DHPG-Induced Nocifensive Behaviors are Reduced in mGlu5 KO Mice: Sensitivity to the group I mGluR agonist DHPG was decreased in KO mice as measured both by spontaneous nocifensive behavior following an intrathecal DHPG injection (**Figure 3a**), and by the development of hypersensitivity to a thermal stimulus following ipsilateral intraplantar injection of DHPG (**Figure 3b**). mGlu5 KO mice did not develop thermal hypersensitivity following DHPG injection, whereas hypersensitivity developed in WT mice at multiple time points. No changes in thermal sensitivity were observed in the contralateral paw of either genotype following intraplantar DHPG injection (data not shown).

Figure 3: DHPG-Induced Spontaneous Nocifensive Behavior and Thermal Hypersensitivity is Reduced in mGlu5 KO Mice

A) Wildtype mice have significantly greater nocifensive behavior than their KO littermates following intrathecal DHPG injection (2-Way ANOVA Main Effect of Genotype *** = p=0.0004; n = 4-5 mice per group). B) Thermal hypersensitivity following DHPG is not observed in mGlu5 KO mice, but is observed in their WT littermates (2-Way ANOVA Main Effect of Genotype *** = p<0.0001; n = 8 mice per group). Paw withdrawal latencies of WT mice were significantly decreased from baseline at multiple time points (1-Way ANOVA Main Effect p=0.02). No time points were significantly different from baseline in KO mice. Paw withdrawal latencies of the paw contralateral to intraplantar DHPG injection were not significantly different (2-Way ANOVA Main Effect of Genotype p=0.90). *, **, and *** = p < 0.05, 0.01, and 0.001 WT compared to KO; †, †† = p<0.05, 0.01 WT time point compared to baseline.



Formalin-Induced Nocifensive Behavior is Reduced in mGlu5 KO Mice: Nocifensive behavior, as measured by time spent licking or lifting the injected paw, was significantly decreased in mGlu5 KO mice as compared to WT littermates (**Figure 4a**). In addition, the time spent licking or lifting was significantly decreased when summed from 0 to 10 minutes (Phase 1, **Figure 4b**) and from 10 to 60 minutes (Phase 2, **Figure 4c**).

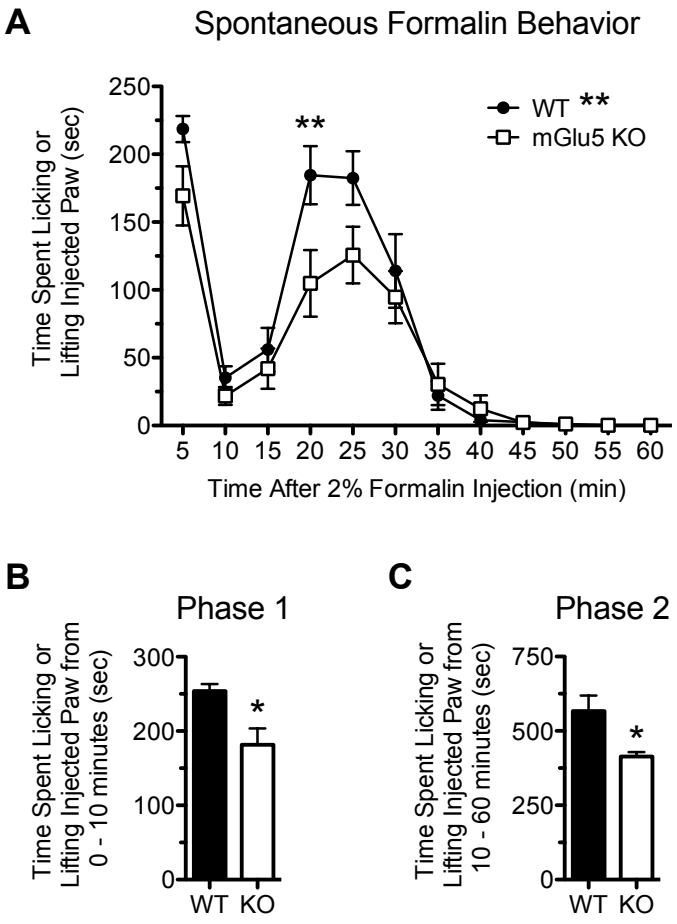


Figure 4: Decreased Nocifensive Behavior Following Intraplantar Formalin Injection in mGlu5 KO Mice.

A) Wildtype mice have significantly greater nocifensive behavior following intraplantar formalin injection as compared to their KO littermates (2-Way ANOVA Main Effect of Genotype ** = $p=0.0023$; 20 minute time point Bonferroni post-test ** = $p<0.01$; n = 5-6 mice per group). The sum of time spent licking or licking in both B) Phase 1 (0-10 minutes; unpaired t-test * = $p=0.02$) and C) Phase 2 (10-60 minutes; unpaired t-test * = $p=0.01$) was significantly less in mGlu5 KO mice.

CFA-Induced Nocifensive Behavior is Reduced in mGlu5 KO Mice: Intraplantar CFA injection induced a unilateral hypersensitivity to mechanical stimuli in both WT and mGlu5 KO mice. However, in mGlu5 KO mice recovery to baseline levels occurred within 1 day, whereas WT mice never recovered to baseline levels (**Figure 5a**). The duration of hypersensitivity to mechanical stimuli was significantly shorter in mGlu5 KO mice compared to WT littermates. No hypersensitivity developed to mechanical stimuli in the contralateral paw of mice from either genotype (data not shown).

Intraplantar CFA injection also induced a unilateral hypersensitivity to thermal stimuli in both WT and mGlu5 KO mice. This hypersensitivity was shorter lived than that to mechanical stimuli and both genotypes had recovered to levels not significantly different from baseline after 120 minutes (WT mice after 60 minutes). Genotype did not affect the development or recovery from thermal hypersensitivity (**Figure 5b**). No hypersensitivity developed to thermal stimuli in the contralateral paw of mice from either genotype (data not shown).

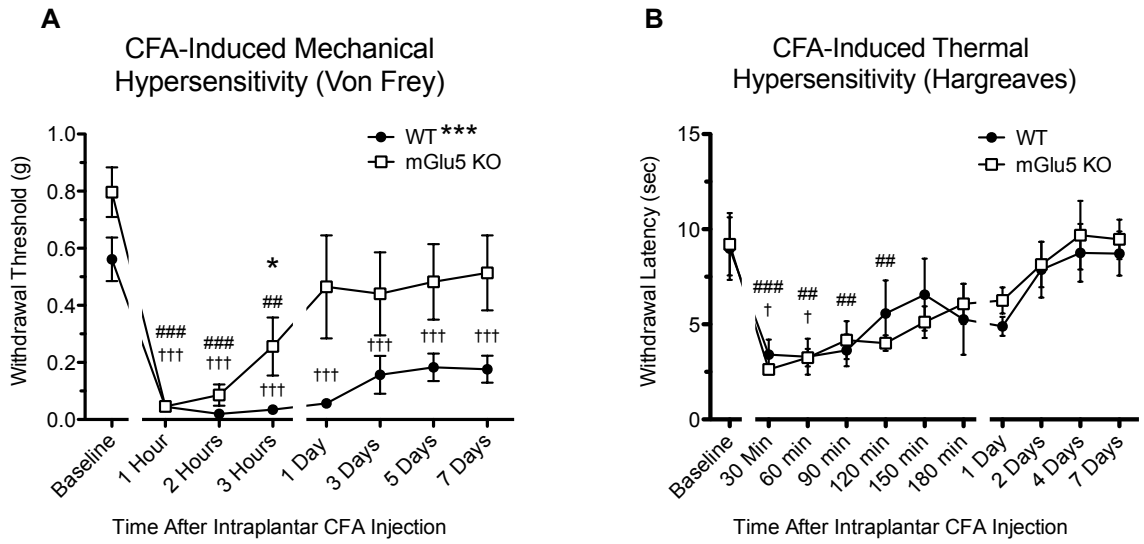


Figure 5: CFA-Induced Mechanical Hypersensitivity is Reduced in mGlu5 KO Mice

a) mGlu5 KO mice have significantly faster recovery from CFA-induced mechanical hypersensitivity compared to their WT littermates (2-Way ANOVA Main Effect of Genotype *** = $p<0.0001$; n = 10 mice per group). Paw withdrawal latencies of WT and KO mice were significantly decreased from baseline at multiple time points (1-Way ANOVA Main Effect $p<0.0001$ and $p=0.0003$ respectively). Paw withdrawal thresholds of the paw contralateral to intraplantar CFA injection were not significantly different (2-Way ANOVA Main Effect of Genotype $p=0.73$). b) CFA-induced thermal hypersensitivity was not different in WT and mGlu5 KO mice (2-Way ANOVA Main Effect of Genotype $p=0.86$; n = 7 mice per group). Paw withdrawal latencies of WT and KO mice were significantly decreased from baseline at multiple time points (1-Way ANOVA Main Effect $p=0.01$ and $p<0.0001$ respectively). Paw withdrawal latencies of the paw contralateral to intraplantar CFA injection were not significantly different (2-Way ANOVA Main Effect of Genotype $p=0.53$).

*, *** = $p < 0.05$, 0.001 WT compared to KO; †, ††† = $p<0.05$, 0.001 WT time point compared to baseline; ## , ### = $p<0.01$, 0.001 KO time point compared to baseline.

Neuropathic Pain Models in mGlu5 KO Mice: Hypersensitivity to evaporative cooling was induced in mGlu5 KO mice and their WT littermates as compared to their respective baselines at all time points measured post chronic constriction injury (**Figure 6a**). However hypersensitivity to evaporative cooling was significantly less in mGlu5 KO mice compared to WT littermates. No hypersensitivity developed to acetone in the contralateral paw of mice from either genotype (data not shown).

Spared Nerve Injury induced mechanical hypersensitivity in both mGlu5 KO mice and their WT littermates compared to their respective baselines at all time points measured post nerve injury (**Figure 6b**). There were no differences attributable to genotype. Hypersensitivity was not induced in the paw contralateral to injury (data not shown).

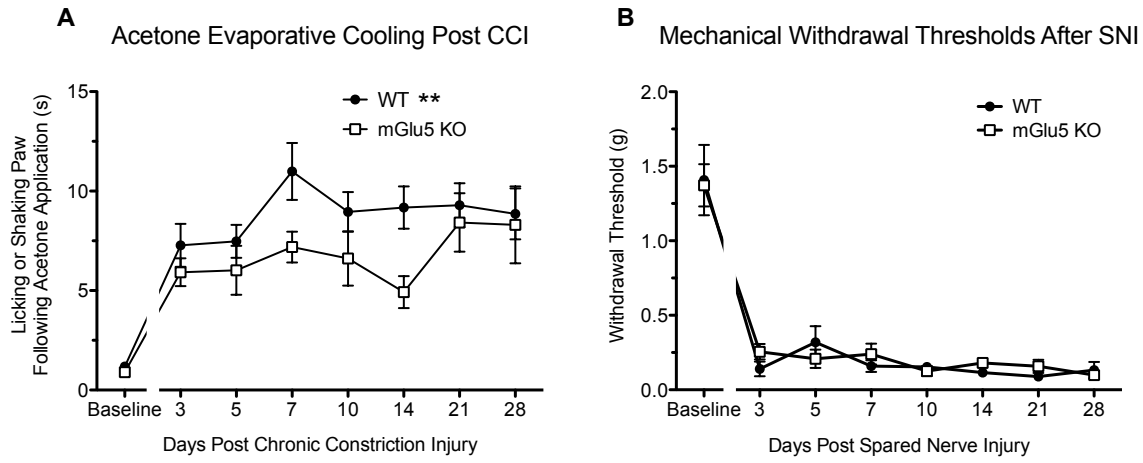


Figure 6: Neuropathic Pain in mGlu5 KO Mice

A) mGlu5 KO mice exhibit significantly less licking or lifting following acetone application (2-Way ANOVA Main Effect of Genotype ** = $p<0.01$; n = 10 - 13 mice per group). Licking and lifting was significantly greater than baseline at all time points for both genotypes (WT and KO 1-Way ANOVA Main Effect $p<0.0001$ and $=0.0002$ respectively). B) mGlu5 KO and WT both exhibited mechanical hypersensitivity after spared nerve injury. No differences were attributable to genotype (2-Way ANOVA Main Effect of Genotype $p=0.80$; n = 4 - 6 mice per group). Mechanical sensitivity was significantly less than baseline at all time points for both genotypes (Both WT and KO 1-Way ANOVA Main Effect $p<0.0001$).

DISCUSSION

The analgesic properties of mGlu5 antagonists have been previously reported (Bhave et al., 2001; Walker et al., 2001a; Zhu et al., 2004). However unambiguous determination of the role mGlu5 plays in the development and maintenance of chronic pain has been hindered by questionable antagonist selectivity. Here I present data from mGlu5 KO mice that show that mGlu5 is required for the full expression of pain-related behaviors. When compared to WT littermates mGlu5 KO mice were found to have reduced nociceptive behaviors in the formalin test and following CFA-induced mechanical hypersensitivity. These data are in agreement with those from antagonist studies. Coupled with the findings presented here that the pro-algesic effects of the group I mGluR agonist DHPG are markedly reduced in mGlu5 KO mice, these findings strongly support the hypothesis that mGlu5 plays a role in nociception. It is interesting that baseline sensitivity of mGlu5 KO mice to thermal and mechanical stimuli were unchanged, suggesting that mGlu5 is only required for the development and maintenance of hypersensitivity and not normal sensation itself.

Some of the findings from mGlu5 KO mice presented here are at odds with those obtained from antagonist studies, however. While the results from the formalin test and the studies of CFA-induced mechanical hypersensitivity recapitulate the effects of MPEP (Zhu et al., 2004; Walker et al., 2001a), the findings that CFA-induced thermal hypersensitivity, and mechanical hypersensitivity post-SNI are the same in mGlu5 KO mice as their wildtype littermates is at odds with results obtained using MPEP (Zhu et al.,

2004). This may be due to the fact that previous studies using MPEP were conducted using rats while the studies here use mice; alternatively it could suggest that MPEP's analgesic effects are off-target for mGlu5. A direct assessment of the analgesic effects of MPEP in mGlu5 KO mice is presented in Chapter 3.

The mice described in this chapter have a global deletion of mGlu5. While these KO mice have reductions in pain-related behaviors, mGlu5 is expressed at synapses throughout the pain neuraxis (Varney and Gereau, 2002), and the global nature of this deletion precludes the determination of the relative contribution of mGlu5 expressed at different synapses to nociceptive signal processing. DHPG is proalgesic when administered into the periphery, spinal cord, and higher brain structures and MPEP administration to all of these sites attenuates nociceptive behaviors (Bhave et al., 2001; Karim et al., 2001; Kolber et al., 2010). Therefore, it is possible that activation of mGlu5 at all levels of the pain neuraxis plays a role in nociception. In Chapter 5 I will report on findings from mice in which mGlu5 was selectively deleted only in peripheral nociceptors to test the hypothesis that peripheral mGlu5 is required for the full expression of pain-related behaviors.

Results from the experiments discussed in this chapter suggest that mGlu5 might be a druggable target for the treatment of pain. In Chapter 3 I will present data on the analgesic properties of the mGlu5 antagonist fenobam, which has been tested in human subjects for the treatment of anxiety. Fenobam has been found to have a good safety profile, and thus could theoretically represent an mGlu5 antagonist suitable for treating

pain in human patients. Finally, mGlu5 antagonists will only be useful for the treatment of human pain conditions if they also have acceptable side-effects profiles. In Chapter 4 I assess the possibility that antagonism of mGlu5 may affect locomotion and motor coordination (Zhu et al., 2004), which would unfortunately represent potential deleterious effects that could reduce the utility of mGlu5 antagonists as analgesics.

Chapter 3

The mGlu5 antagonist fenobam is analgesic and has improved in vivo selectivity as compared to the prototypical antagonist MPEP.

INTRODUCTION

As discussed in chapter 2, mGlu5 KO mice have reduced sensitivity to painful stimuli. Thus, mGlu5 represents a potential molecular target for the therapeutic treatment of pain. However, past studies of mGlu5 antagonists have been primarily limited to pre-clinical animal models. Research into the efficacy of mGlu5 antagonists in the treatment of human pain conditions would be greatly enhanced by an antagonist that has been shown to be safe and pharmacologically active in humans. Recently, researchers at Hoffmann-La Roche demonstrated that the clinically validated non-benzodiazepine anxiolytic fenobam [N-(3-chlorophenyl)-N'-(4,5-dihydro-1-methyl-4-oxo-1H-imidazole-2-yl)urea] is a highly potent and selective antagonist of mGlu5 (Porter et al., 2005). McNeil Laboratories originally developed Fenobam as a potential anxiolytic in the 1970s and 80s, but with a then unknown molecular target. It was found to be effective at treating anxiety in a double-blinded placebo-controlled clinical trial (Pecknold et al., 1982). In that trial, and two additional trials (Pecknold et al., 1980; Lapierre and Oyewumi, 1982), fenobam was reported to have a good safety profile with no oversedation, no muscle relaxation, and no interaction with ethanol. However in a different phase II clinical trial (Friedmann et al., 1980) fenobam was reported to have both psychostimulant side effects and be ineffective as an axiolytic. In the early 1980s, further development of the molecule as an anxiolytic by McNeil was discontinued. Then in 2005, a functional high-throughput screen and subsequent characterization by Porter, et al. identified fenobam as a potent, non-competitive, mGlu5 selective antagonist, acting at

an allosteric modulatory site shared with the prototypical mGlu5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) (Gasparini et al., 1999). Interestingly, in addition to its anxiolytic efficacy (Ballard et al., 2005) MPEP has also been reported effective in the treatment of animal models of both Fragile X syndrome (FXS) (Yan et al., 2005) and chronic pain (Zhu et al., 2004). Fenobam was recently tested as a potential clinical therapeutic for FXS in humans, where it was shown to possess beneficial clinical effect without significant adverse effects (Berry-Kravis et al., 2009). However, the efficacy of fenobam in the treatment of pain remains untested.

Given the interest in mGlu5 as a target for inflammatory pain and the recent demonstration of fenobam's efficacy as a clinically validated mGlu5 antagonist with a good safety profile, I sought to compare fenobam's analgesic efficacy to that of MPEP in several in vivo models of inflammatory pain. Here I show that fenobam, like MPEP, can prevent formalin-induced spontaneous pain-related behaviors and reduce Complete Freund's Adjuvant (CFA)-induced thermal hypersensitivity. In addition, fenobam was also found to prevent spontaneous pain-related behaviors induced by the algogenic TRPA1 agonist mustard oil (Jordt et al., 2004). Previous pharmacokinetic analysis of fenobam in humans has indicated variable plasma levels (Berry-Kravis et al., 2009), thus I also sought to characterize the bioavailability of fenobam in both mouse plasma and brain tissue. Studies were also tested to determine whether tolerance to the analgesic effects of fenobam develop following repeat dosing. In order to establish selectivity for its analgesic effects on mGlu5, I also assessed the pain behaviors of mGlu5 KO mice that

were treated with either fenobam or MPEP. Finally, as will be discussed in detail in Chapter 4, I also assessed fenobam in a battery of tests designed to look for potential undesirable effects.

METHODS

Animals: Experiments were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Animal Care and Use Committee of Washington University School of Medicine. Male Swiss-Webster mice (29.5 – 43 g) were purchased from Taconic. Male C57BL/6 mice used in metabolism experiments (20.0 – 29.5 g) were purchased from Jackson Labs. For experiments involving mice lacking mGlu5 (mGlu5 KO; 16.5 – 22.5 g), animals were bred inhouse on a C57BL/6 background and compared to WT littermates (Lu et al., 1997). For experiments involving KO animals the experimenter was blinded to genotype. All other C57BL/6 WT mice used in behavioral experiments were also derived from this colony. Blinding to genotype was accomplished by using coded ear tag identification numbers and only breaking the code at the end of the experiment. Genotyping of mice bred inhouse was performed using standard PCR techniques as described in Chapter 2. All mice were group housed on a 12/12-light/dark schedule with *ad libitum* access to food and water.

Chemicals and Reagents: Fenobam and MPEP were purchased from Tocris (Ellisville, MO). Both compounds were dissolved in DMSO (100%) (Sigma-Aldrich, St. Louis, MO) on the day of experiment. All intraperitoneal (i.p.) injection volumes were 20 microliters. Throughout all experiments the investigator was blinded to pharmacological treatment. Midazolam was purchased from Cerilliant (Round Rock, TX) and used as the HPLC/MS

internal standard. All other reagents were HPLC grade and purchased from Sigma-Aldrich Co. (St. Louis, MO).

General behavioral testing conditions: Behavioral tests were conducted in the same general manner as described in Chapter 2.

Spontaneous Formalin-Induced Nocifensive Behavior: Mice were acclimated in a transparent Plexiglas box (25L x 13W x 13H cm) for 2 hours prior to any drug injection. Animals were then pretreated by i.p. injection with vehicle, MPEP, or fenobam. All Swiss Webster mice were pretreated 30 minutes prior to formalin injection. Strain differences in responsiveness to fenobam were noted in pilot studies and thus C57BL/6 mice were initially pretreated either 5 or 30 minutes prior to formalin injection to determine the optimal time for pretreatment in this strain (n = 4-5 per group). In all subsequent formalin tests involving C57BL/6 mice, including those performed with mGlu5 KO mice, vehicle or drugs were injected 5 minutes prior to formalin injection. Ten microliters of dilute formalin solution (Sigma, St. Louis, MO) was injected subcutaneously into the plantar surface of the right hind paw. Due to strain differences in formalin sensitivity (Mogil et al., 1999), the concentration of formalin injected into Swiss-Webster mice and C57BL/6 mice was 5% and 2% respectively. The time spent in nociceptive behavior, defined as licking, lifting, or flicking of the injected paw, was scored in five-minute intervals for one hour beginning immediately after paw injection. For experiments comparing fenobam to

MPEP in Swiss Webster mice, 30 mg/kg of each drug was administered (n=7 per group). For experiments intended to develop a dose response curve for fenobam in Swiss Webster mice, 3, 10, 30, or 100 mg/kg of fenobam was injected (n = 7-11 per group). For experiments involving the testing of fenobam in C57BL/6 WT and mGlu5 knockout mice 30 mg/kg of fenobam was injected (n=4-6 per group). For experiments involving the testing of MPEP in mGlu5 KO mice 30 mg/kg MPEP was injected (n = 6-9 per group).

Spontaneous Formalin-Induced Nocifensive Behavior following Chronic Fenobam Treatment: Male Swiss Webster mice mice were injected i.p. once per day for 5 days with 30 mg/kg of fenobam or vehicle (DMSO). On the 6th mice were placed in transparent Plexiglas boxes (10L x 10W x 15H cm) on a glass surface and mice from each group (chronic fenobam and chronic vehicle) were both injected with either fenobam or vehicle, followed 5 minutes later by intraplantar formalin injection. Thus, eight separate groups were analyzed, 1, 2) chronic vehicle / acute vehicle; 3, 4) chronic fenobam / acute vehicle; 5, 6) chronic vehicle, acute fenobam; 7,8) chronic fenobam, acute fenobam; with chronicity defined as 5 days. The time spent in nocifensive behavior was scored in five-minute intervals for 45 minutes after paw injection as described above.

Spontaneous Mustard Oil-Induced Nocifensive Behavior: Mustard oil-induced nocifensive behavior was performed in the same manner as that described for the formalin test, except 0.5% mustard oil was injected subcutaneously instead of formalin.

In addition, behavioral responses were only scored for 30 minutes post mustard oil injection. Fenobam (30 mg/kg) or vehicle (DMSO) were injected 30 minutes prior to scoring mustard-oil induced behaviors (n= 4 per group).

Complete Freund's Adjuvant (CFA) Induced Thermal Hypersensitivity: Thermal hypersensitivity was measured in a manner similar to that described in Chapter 2. Briefly, Swiss Webster (n = 8 per group) mice were placed in individual Plexiglas containers (10L x 10W x 15H cm) on an elevated glass platform. A continuous radiant heat source was delivered through the glass onto the surface of each hind paw (IITC Life Sciences, Woodland Hills, CA), and the latency for animals to withdraw their paw was measured. The active intensity of the heat source was set to 21%. Five baseline measurements were obtained 15 minutes apart and averaged. Immediately following baseline CFA (10 microliters; 1 mg/ml) was injected into the plantar surface of the right hind paw. Paw withdrawal latency to the thermal stimulus ($PWL_{Thermal}$) was assessed 48 hours after CFA injection. One hour following assessment of post-CFA $PWL_{Thermal}$, animals were injected i.p. with fenobam (30 mg/kg) or vehicle. $PWL_{Thermal}$ was then assessed 30 and 60 minutes post drug injection.

Data Analysis: Statistical analysis of behavioral data was performed in the same manner as described in Chapter 2.

Method for Quantification of Fenobam in Mouse Brain and Plasma

Plasma and Brain Tissue Collection: Wild type Swiss Webster and C57BL/6 (n = 4 per group) mice were injected with fenobam (3, 10, or 30 mg/kg, i.p.) and administered an overdose of sodium pentobarbital (75 mg/kg) 5, 30, or 55 minutes after fenobam injection. Whole blood was obtained by transcutaneous cardiac puncture and plasma was separated via centrifugation (5 min, 4 °C, 14K G) in plasma separator tubes with lithium heparin (BD Microtainer). Immediately post centrifugation plasma was frozen using liquid nitrogen and stored at -80 °C. Brains were dissected and immediately frozen in liquid nitrogen and stored at -80 °C. Whole brain homogenates were obtained by adding milliQ water to brain tissue in a 2:1 (v:w) ratio and sonicating using a Branson Sonifier 150. Brain homogenates were immediately refrozen on dry ice.

General Instrumentation: Fenobam was quantitated using LC/MS/MS. Calibrators were prepared in a matrix matching the samples (brain homogenates or plasma). Midazolam was used as the internal standard. Instrumental analysis was performed on an API 4000QTRAP triple-quadropole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA), equipped with a Turbo IonSpray Source. The Agilent 1100 HPLC system (Waldbronn, Germany) included a binary pump, a thermostatted well-plate autosampler, and a column thermostat. An external two-way Valco valve was utilized to direct HPLC flow to waste before and after column elution of analytes of interest. Chromatographic separation was performed on a SymmetryShield RP18 analytical

(3.5µm, 2.1 x 30mm) column (Waters Corp., Milford, MA, USA) with a C18 guard (5µm, 2 x 10mm) column (Varian, Lake Forest, CA, USA) at 30°C. Before each injection, the needle was washed with methanol. Separate methods were used for determining fenobam in brain tissue and plasma.

HPLC and Mass Spec Conditions for Quantification of Fenobam in Brain Tissue: Mobile phase A was 20mM ammonium formate buffer, pH 5.7 and mobile phase B was methanol. Mobile phase was delivered at an initial condition of 2% B and a flow rate of 0.5ml/min with the following time program: 2% B is held for 0.1 minute, followed by a linear gradient to 100% B between 0.1 and 1.0 minute. B is held at 100% for 0.1 minute, and then brought back down to initial condition of 2% between 1.1 and 2.1 minutes. The column is re-equilibrated with 2% B from 2.1 to 5.0 minutes. Under these conditions, the retention time for fenobam was 3.3 minutes and for midazolam was 3.4 minutes. The injection volume was 10 microliters. Both Q1 and Q3 quadrupoles of the mass spectrometer were optimized to unit mass resolution, and the conditions were optimized for each analyte. The instrument was operated in positive-ion mode with an ion spray voltage of 5500 volts. The curtain gas was set at 20 psi, ion source gas 1 and 2 at 20 psi, and the collision gas on “high”. The transitions monitored for each analyte, along with the analyte specific parameters are listed in Table 1.

Calibration and Sample preparation: 50 microliters of each homogenized sample was pipetted into discrete wells of a 96-well 2.2ml plate. Twenty-five microliters of internal standard (12.5ng midazolam) was added to each sample, followed by 200 microliters of acetonitrile. The plate was capped and vortexed, and then centrifuged at 3000 rpm for 10 minutes. The supernatants were transferred to a 96-well autosampler plate, and 10 microliters was injected. Calibrators and quality control samples were prepared along with experimental samples.

Calibrators, quality controls and internal standard samples: A methanolic solution of fenobam was prepared at 1mg/mL. Dilutions from this stock standard were prepared and used to make calibrator and quality control (QC) samples in brain homogenate. Brain homogenate calibrators contained 0.5, 2.5, 5, 10, 15, 20, 30 and 40µg/g. Brain homogenate QCs were made at 2.5 and 20µg/g.

HPLC and Mass Spec Conditions for Quantification of Fenobam in Plasma: Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Mobile phase was delivered at an initial condition of 5% B and a flow rate of 0.4ml/min with the following time program: linear gradient between 5 and 60% B for 1.0 minute followed by a sharp gradient to 100% B for 0.2 minute and hold at 100% B for 0.4 min; mobile phase composition is then brought back down to initial condition of 5% between 1.6 and 2.1 minutes. The column is re-equilibrated with 5% B from 2.1 to 5.5

minutes. Under these conditions, the retention time for fenobam was 3.4 minutes and for midazolam was 3.0 minutes. The injection volume was 20 microliters. Both Q1 and Q3 quadrupoles of the mass spectrometer were optimized to low and unit mass resolution respectively. The instrument was operated in positive-ion mode with an ion spray voltage of 5100 volts. The curtain gas was set at 20 psi, ion source gas 1 and 2 at 40 and 50 psi respectively, and the collision gas on high. The transitions monitored for each analyte, along with the analyte specific parameters are listed in Table 2.

Calibration and Sample preparation: Mouse plasma samples were thawed, homogenized and aliquots of 25 microliters were transferred into a 96-well plate. Precipitation was performed using 100 microliters of acetonitrile, which contained 50ng/ml of midazolam (internal standard). The plate was capped and vortexed, and then centrifuged at 3000 rpm for 10 minutes. The supernatants were transferred to a 96-well autosampler plate, and 20 microliters were injected for analysis. Calibrators and quality control samples were prepared along with experimental samples.

Calibrators, quality controls and internal standard samples: A methanolic solution of fenobam was prepared at 1mg/ml. Dilutions from this stock standard were prepared and used to make calibrator (6.0 to 16,000 ng/ml, 10 concentrations) and quality control (QC) samples (2 concentrations) in human plasma. Preliminary experiments using mouse and human plasma had similar LC/MS/MS results.

Table 1: HPLC-mass spectrometric acquisition parameters for brain tissue

Q1 Mass	Q3 Mass	Time	Declustering Potential	Exit Potential	Collision Energy	Collision Cell Exit Potential
amu		*ms*			*V*	
267.1	114	150	76	10	21	18
267.1	140	150	76	10	25	22
326.2	249.3	150	126	10	53	16
326.2	291.2	150	126	10	37	6

Table 2: HPLC-mass spectrometric acquisition parameters for plasma

Q1 Mass	Q3 Mass	Time	Declustering Potential	Exit Potential	Collision Energy	Collision Cell Exit Potential
amu		*ms*			*V*	
267.1	114	30	76	10	21	18
267.1	140	30	76	10	25	22
326.2	223.2	30	90	10	50	16
326.1	291.2	30	90	10	40	14

RESULTS

Effects of Fenobam and MPEP on Spontaneous Formalin Behavior in WT Mice: I compared the effects of fenobam to MPEP, an mGlu5 antagonist that has been previously demonstrated to reduce spontaneous behavior during the formalin test. Right hind paw injection of formalin resulted in a characteristic biphasic response (**Figure 1a**). Pre-treatment with both fenobam (30 mg/kg i.p.) and MPEP (30 mg/kg i.p.) significantly reduced the time Swiss Webster mice spent licking or lifting the formalin injected paw during the second phase (**Figure 1c**). In addition pre-treatment with fenobam resulted in a reduction in the time spent licking or lifting during the first phase (**Figure 1b**).

I next examined the effects of four different doses of fenobam (3, 10, 30, and 100 mg/kg i.p.) on nociceptive scores during the formalin test (**Figure 2a**). Pre-treatment of Swiss Webster mice with 30 and 100 mg/kg fenobam 30 minutes prior to intra-plantar formalin injection significantly reduced the time mice spent licking or lifting the injected paw during both the first (**Figure 2b**) and second phase (**Figure 2c**).

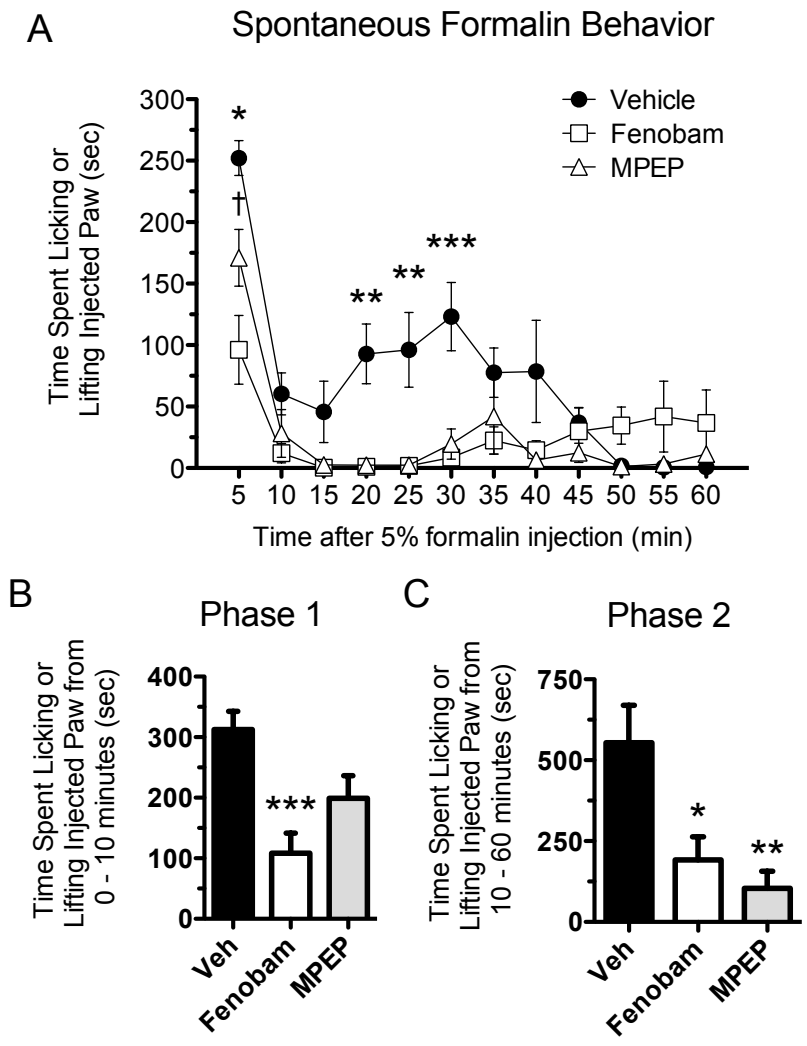


Figure 1: Spontaneous formalin behavior is reduced following pretreatment with fenobam or MPEP in Swiss Webster mice.

A) When administered 30 min prior to intraplantar formalin injection both Fenobam (30 mg/kg, i.p.) and MPEP (30 mg/kg, i.p.) significantly decrease the time spent licking or lifting the injected paw (2-Way ANOVA Main Effect of Drug $p< 0.0001$). Asterisks indicate time points where both drug treatments are significantly different from vehicle (n = 7 per group; *, **, *** = $p< 0.05$, 0.01, 0.001 respectively). Fenobam treated animals were significantly different from MPEP at the 5 min time point († = $p < 0.05$) B, C) Total time spent licking or lifting in the first (0-10 minutes; 1-Way ANOVA Main Effect of drug $p=0.0018$) and second phase (10-60 minutes; 1-Way ANOVA Main Effect of drug $p=0.0033$) respectively.

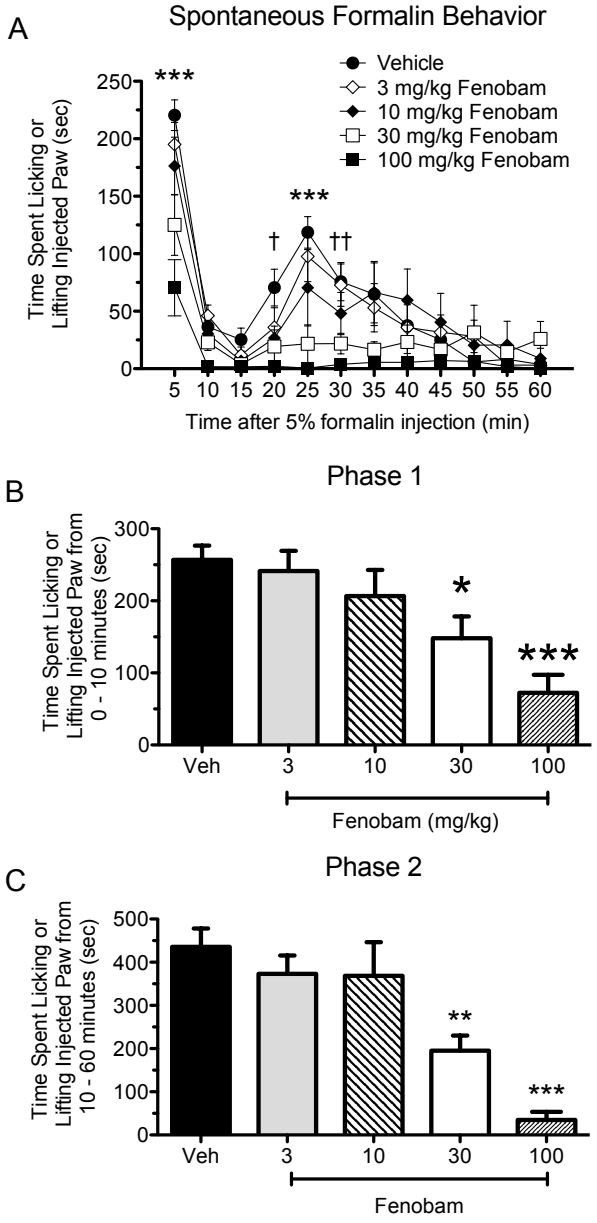


Figure 2: Effect of fenobam (3, 10, 30, or 100 mg/kg, i.p) on spontaneous formalin behavior in Swiss Webster mice.

A) When Fenobam is administered 30 min prior to formalin, a minimum dose of 30 mg/kg, i.p. is required to see a significant effect (n = 7-11 per group; 2-Way ANOVA Main Effect of Fenobam $p< 0.0001$; *** = $p < 0.001$ for vehicle compared to 30 mg/kg and 100 mg/kg; †, †† = $p < 0.05$, 0.01 respectively for vehicle compared to 100 mg/kg). (B, C) Total time spent licking or lifting in the first (0-10 minutes; 1-Way ANOVA Main Effect of drug $p=0.0002$) and second phase (10-60 minutes; 1-Way ANOVA Main Effect of drug $p<0.0001$). Dunnett's Post Test *, **, *** = $p< 0.05$, 0.01, 0.001 respectively as compared to vehicle.

The C57BL/6 strain of mice is often used in the generation of KO animals, and it is the background strain of the mGlu5 KO mice that I used in this thesis. I therefore examined the effects of fenobam on spontaneous formalin behavior in this strain. In Swiss Webster mice pretreatment with fenobam was performed 30 minutes prior to formalin injection. When C57BL/6 mice were pretreated with fenobam (30 mg/kg) 30 minutes prior to formalin injection there was a reduction in formalin-induced spontaneous behavior 20 minutes after formalin injection as compared to vehicle, however the total time in the second phase was not found to be different. When C57BL/6 mice were instead pre-treated with fenobam 5 minutes prior to formalin injection a significant reduction in the second phase was observed as compared to both vehicle injected mice and mice injected with fenobam 30 minutes prior to formalin injection (**Figure 3**).

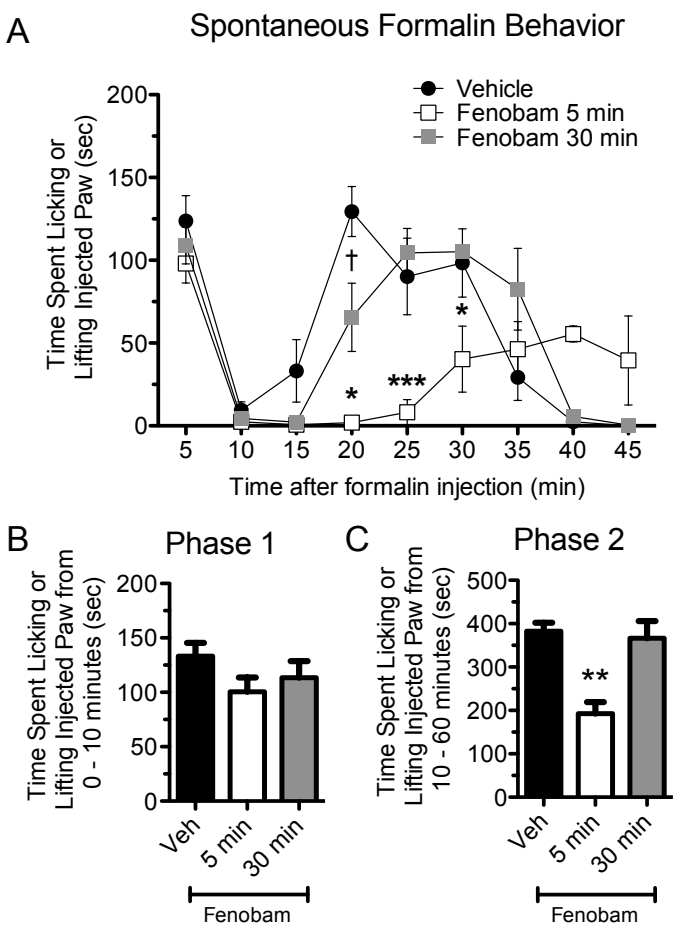


Figure 3: Spontaneous formalin behavior is reduced following pretreatment with fenobam in WT C57BL/6 mice.

A) When administered 30 minutes prior to intraplantar formalin injection fenobam (30 mg/kg, i.p.) significantly decreased the time spent licking or lifting the injected paw as compared to vehicle at 20 minutes post injection (2-Way ANOVA Main Effect of Fenobam $p=0.0007$; † = $p<0.05$). The overall time spent licking or lifting in the second phase was not reduced. When fenobam (30 mg/kg, i.p.) was injected 5 minutes prior to formalin hind-paw injection there was a significant reduction at A) multiple time points (*, *** = $p<0.05$, 0.001) and C) in the total time spent licking or lifting in the second phase (10-60 minutes; 1-Way ANOVA Main Effect of drug $p<0.0015$; Bonferroni Post Test $p< 0.01$) as compared to both vehicle and animals injected with fenobam 30 minutes prior to formalin injection (n = 4-5 per group).

Effects of Fenobam on Spontaneous Mustard Oil-Induced Behavior in WT Mice: Mustard oil (allyl isothiocyanate), the pungent component in wasabi and horseradish, is a potent and selective agonist of the cation channel TRPA1 (Jordt et al., 2004). Right hindpaw injection elicited spontaneous licking and flinching behaviors that were significantly reduced by 30 minute pretreatment with 30 mg/kg fenobam (**Figure 4**).

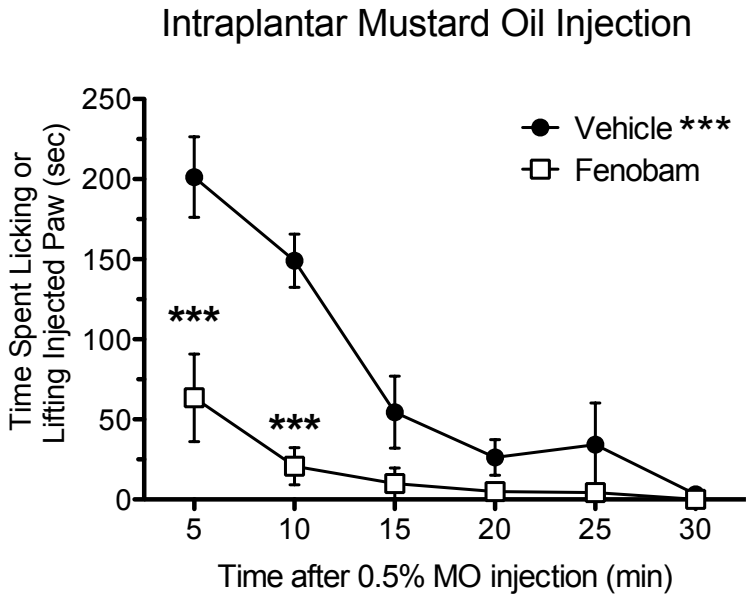


Figure 4: Spontaneous Mustard Oil-induced Behavior is Reduced by Fenobam Pretreatment

Pretreatment with fenobam (30 mg/kg i.p.) reduces spontaneous licking and lifting induced by intraplantar injection of 0.5% mustard oil (n=4 per group). Fenobam or vehicle (DMSO) was injected 30 min prior to MO. injection. (2-Way ANOVA Main Effect of Fenobam $p < 0.0001$; Bonferroni Post-hoc test *** = $p < 0.001$)

Fenobam Reverses CFA-Induced Thermal Hypersensitivity in WT Mice: The effects of fenobam and vehicle on CFA induced thermal hypersensitivity were compared. Forty-eight hours following injection of CFA into the right hind paw, paw withdrawal latency to a thermal stimulus ($PWL_{Thermal}$) relative to baseline was significantly reduced ipsilateral to the injection in both groups (**Fig. 5**). No reduction in $PWL_{Thermal}$ was observed in the contraleral paw in either group. One hour following assessment of the 48-hour-post-CFA $PWL_{Thermal}$, mice were injected i.p with vehicle or fenobam (30 mg/kg). Fenobam, but not vehicle, significantly increased $PWL_{Thermal}$ measured 30 and 60 minutes following drug injection as compared to their respective 48-hour-post-CFA time point (**Figure 5**). In the fenobam treated animals the post-drug $PWL_{Thermal}$ returned to a level that was not significantly different from baseline, however the $PWL_{Thermal}$ of the vehicle treated animals remained significantly different from baseline 30 minutes after vehicle injection (**Figure 5**). No differences were observed in the contralateral paw of any group between baseline, pre-drug, or post-drug $PTWL_{Thermal}$ (data not shown).

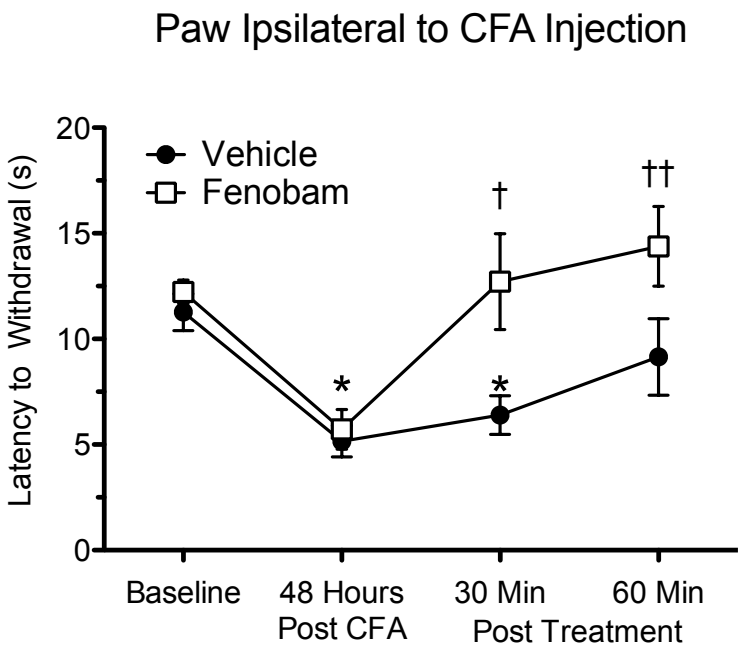


Figure 5: Fenobam relieves established CFA-induced thermal hypersensitivity.

Paw withdrawal latency to a thermal stimulus was reduced 48 hours after an ipsilateral CFA injection, as compared to baseline. Fenobam, but not vehicle, significantly increased thermal withdrawal latency 30 and 60 minutes after treatment in the ipsilateral paw. (1-Way ANOVA Main Effect of Veh $p = 0.0039$; Main Effect of Fenobam $p = 0.0031$; * = $p<0.05$ both groups as compared to baseline; †,†† = $p< 0.05$, 0.01 Fenobam as compared to 48 hours post CFA; n = 8 per group).

Fenobam Disposition in Plasma and Brain Tissue: Fenobam disposition was examined in both brain and plasma from C57BL/6 and Swiss Webster mice. Fenobam was readily detectable in plasma and brain tissue five minutes after i.p. injection in both strains (**Figure 6a,b**), and concentrations decreased thereafter. Fenobam (3 and 10 mg/kg) was largely cleared from both plasma and brain tissue after 30 minutes, while the highest dose (30 mg/kg) remained detectable 30 minutes after injection, but was largely cleared after 55 minutes. A brain:plasma ratio (g/g / g/ml) was calculated for the 5 minute time point for all three doses (**Figure 6c**), and was found to be significantly different in both strains when the 3 mg/kg and 30 mg/kg dose were compared.

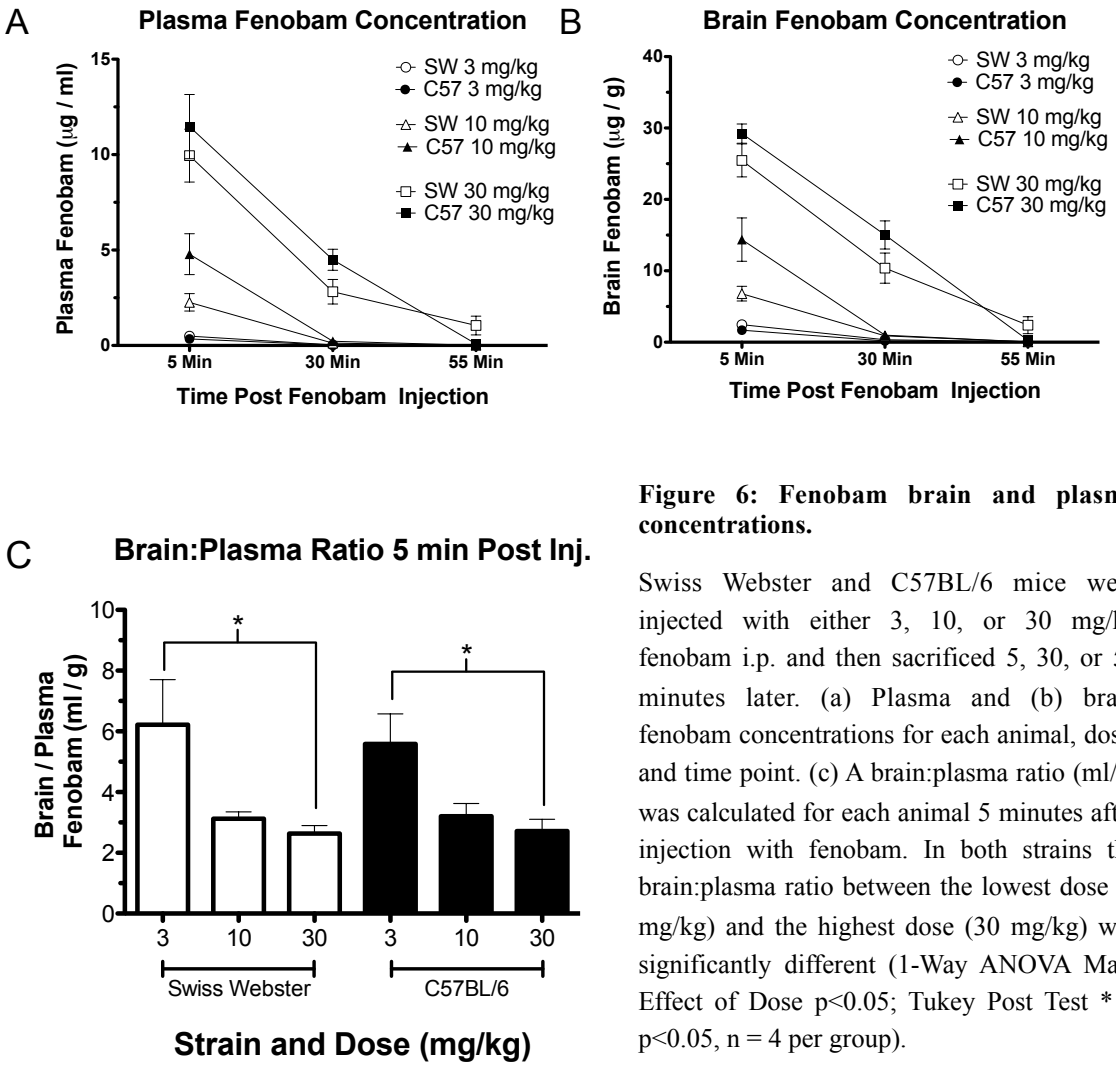


Figure 6: Fenobam brain and plasma concentrations.

Swiss Webster and C57BL/6 mice were injected with either 3, 10, or 30 mg/kg fenobam i.p. and then sacrificed 5, 30, or 55 minutes later. (a) Plasma and (b) brain fenobam concentrations for each animal, dose, and time point. (c) A brain:plasma ratio (ml/g) was calculated for each animal 5 minutes after injection with fenobam. In both strains the brain:plasma ratio between the lowest dose (3 mg/kg) and the highest dose (30 mg/kg) was significantly different (1-Way ANOVA Main Effect of Dose $p<0.05$; Tukey Post Test * = $p<0.05$, n = 4 per group).

Effects of Fenobam and MPEP on Spontaneous Formalin Behavior in mGlu5 KO Mice: The effects of fenobam and MPEP on spontaneous formalin behavior were assessed in mGlu5 knockout mice in the same manner as described for Swiss Webster WT mice. As KO mice were bred on a C57BL/6 background, the effects of fenobam (30 mg/kg) were also assessed on litermate WT mice. Pre-treatment with fenobam (30 mg/kg, i.p. 5 min. prior to formalin injection) significantly reduced the time WT littermates spent licking or lifting the formalin injected paw in both the first phase and second phase (**Figure. 7 a-c**). However, pretreatment with fenobam (30 mg/kg, i.p) did not alter spontaneous nociceptive behavior following formalin injection in mGlu5 KO mice at any time point (**Figure 7 d-f**). When mGlu5 KO mice were pre-treated with MPEP (30 mg/ kg, i.p.), there was a significant reduction in the time spent licking or lifting the formalin injected paw in the second phase, but not the first (**Figure 7 g-i**).

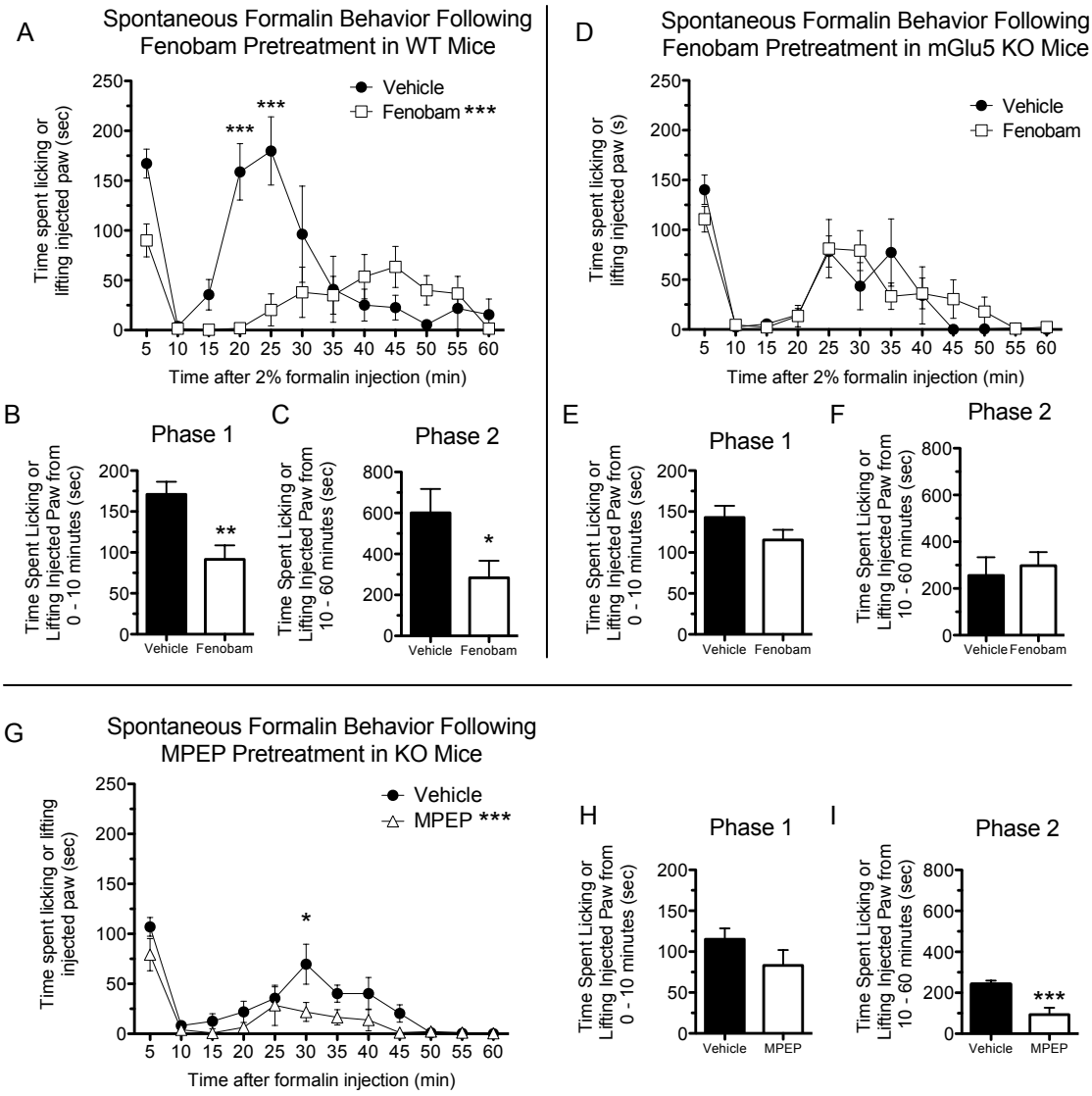


Figure 7: Spontaneous Formalin Behavior is Reduced by MPEP, but not Fenobam in mGlu5 KO Mice.

In C57 WT mice (a-c), but not mGlu5 KO litermates (d-f), fenobam (30 mg/kg i.p.) administered 5 min prior to intraplantar formalin injection significantly decreased the time spent licking of lifting the injected paw (2-way ANOVA Main Effect of Fenobam *** = $p < 0.0001$ in WT; $p = 0.8513$ in KO; Bonferroni Post-hoc test *** = $p < 0.001$). Pretreatment with fenobam reduced both the first (unpaired t-test ** = $p = 0.0082$) (b) and second (unpaired t-test $p=0.049$) (c) phases in WT mice. (n = 4-6 per group.) MPEP (g-i) (30 mg/kg, i.p.) also reduced formalin behavior in mGlu5 KO mice when administered 30 min prior to intraplantar formalin injection (2-way ANOVA Main Effect of MPEP *** = $p = 0.0004$). Asterisks indicate time point where MPEP is significantly different from vehicle (n = 6-9 per group; Bonferroni Post-hoc test * = $p < 0.05$). Pretreatment with MPEP reduced the second phase (unpaired t-test *** = $p=0.0009$) (i) but not the first (unpaired t-test $p=0.18$) (i).

Tolerance Does not Develop to the Analgesic Effects of Fenobam: To test whether tolerance to the analgesic effects of fenobam develops over time I injected mice with either vehicle or fenobam (30mg/kg) for five days and then on the sixth day injected half of each group (chronic vehicle and chronic fenobam) with fenobam 5 minutes prior to performing a formalin test. Regardless of whether they had been treated chronically with fenobam or vehicle, mice acutely treated with fenobam demonstrated significantly less spontaneous nocifensive behavior post formalin injection when compared to chronic vehicle mice that were injected acutely with vehicle (**Figure 8a**). Pre-treatment with fenobam (30 mg/kg i.p.) significantly reduced the time mice spent licking or lifting the formalin injected paw during the first (**Figure 8b**) and second phase (**Figure 8c**) compared to 5 day Veh / Veh mice, regardless of fenobam pretreatment.

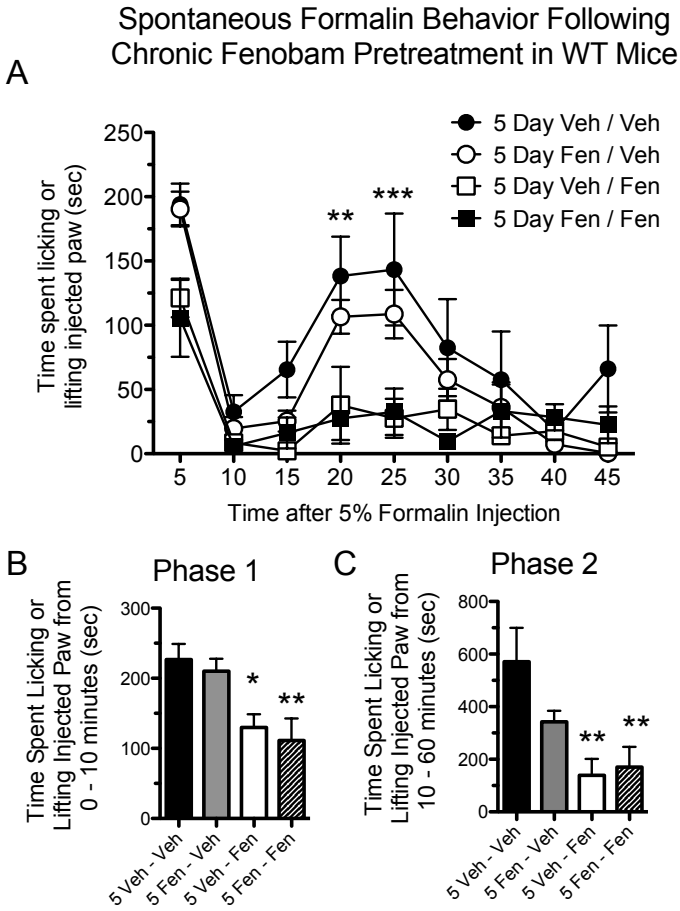


Figure 8: Spontaneous formalin behavior is reduced following five days of pretreatment with fenobam

A) Mice that were administered fenobam or vehicle for 5 days prior to the formalin test demonstrated significantly decreased time spent licking or lifting the injected paw when administered fenobam (30mg/kg) 5 minutes prior to intraplantar formalin injection as compared to mice treated with vehicle for 5 days and vehicle on the day of the experiment (2-Way ANOVA Main Effect of Treatment $p<0.0001$; Bonferroni Post Test **, *** = $p<0.01$, 0.0001 compared to 5 Day Veh/Veh). Both the first phase (b) and the second phase (c) were reduced (1-Way ANOVA Main Effect of Treatment $p=0.003$, $p = 0.004$ respectively). Dunnett's Post Test *, **, *** = $p< 0.05$, 0.01, 0.001 respectively as compared to 5 day vehicle / vehicle mice.

DISCUSSION

Fenobam is a potent and selective mGlu5 antagonist that has anxiolytic properties in both rodents (Porter et al., 2005) and humans (Pecknold et al., 1980, 1982). Multiple groups have reported that pharmacological blockade of mGlu5 is effective at reducing nociception in several rodent pain models (Bhave et al., 2001; Karim et al., 2001; Walker et al., 2001; Zhu et al., 2004), however these studies were the first to report the analgesic properties of fenobam. In the present study I found that i.p. administration of fenobam (30 mg/kg) is analgesic in three mouse models of inflammatory pain, the formalin test, mustard oil induce nocifensive behavior, and the CFA-induced thermal hypersensitivity test. Pretreatment with fenobam reduced the time that mice from two different strains (Swiss Webster and C57BL/6) spent in spontaneous nocifensive behavior during the first and second phase of the formalin test. The effects of fenobam were dose-dependent; 30 mg/kg was the lowest effective dose tested. These findings are similar to those seen when mice are pretreated with the prototypical mGlu5 antagonist MPEP (30 mg/kg). MPEP has been previously shown to reduce spontaneous nocifensive behaviors in the formalin test by several groups, with minimum effective doses in mice ranging from 10 mg/kg (Varty et al., 2005) to 30 mg/kg (Satow, et al., 2008). Comparison between fenobam and MPEP were made at a dose of 30 mg/kg because MPEP has been shown to be effective at 30 mg/kg in reducing formalin induced nocifensive behaviors by multiple groups. These findings suggest that fenobam is capable of reducing both the neurogenic (phase I) and inflammatory (phase II) components of the formalin test. Fenobam was also found to be

effective in reducing the time mice spent in nocifensive behavior following mustard oil injection. As both mustard oil and formalin have been shown to activate the ion channel TRPA1 (Jordt et al., 2004; McNamara et al., 2007), this suggests that mGlu5 may modulate that particular ion channel. In addition fenobam (30mg/kg) was found to relieve established thermal hypersensitivity induced by CFA injection. The description of analgesic efficacy of an mGlu5 antagonist that has also been shown to be pharmacologically active and safe in humans may provide a pharmacological tool for assessing mGlu5 antagonists as analgesics in human clinical trials.

Initial tests were performed in outbred Swiss Webster mice. When attempts were made to begin testing in C57BL/6 mice, a common strain used as a background for KO animals, including the mGlu5 KO mice described here and in Chapter 2, a difference was note in sensitivity to the drug as compared to Swiss Webster mice. Interstrain variability in response to both opiate (Pick et al., 1991) and non-opiate analgesics (Wilson et al., 2003) has been previously reported, and our findings suggest that different strains of mice will also exhibit variable sensitivity to fenobam. It was hypothesized that a difference in fenobam concentration in brain tissue or plasma might underlie the difference in responsiveness between Swiss Webster and C57BL/6 mice, however both strains exhibited nearly identical uptake of fenobam into brain and clearance of fenobam from both brain tissue and plasma at all doses and time points tested. It should be noted that mGlu5 is also expressed in the periphery (Bhave et al., 2001) and the spinal cord (Varney and Gereau, 2002), where potential differences in fenobam concentration were not

assessed. While the brain:plasma ratio of fenobam 5 minutes after i.p. injection was found to vary depending on dose, this variance was noted in both strains and thus cannot account for any observed differences. Finally, disposition studies also demonstrated that fenobam is rapidly cleared from both brain tissue and plasma, such that when administered at 3 or 10 mg/kg the drug is nearly absent 30 minutes after injection. This rapid clearance may explain why the 30 mg/kg dose was required to see analgesic effect in the formalin test.

I also sought to establish that the in vivo anti-nociceptive effects of both MPEP and fenobam were specific for mGlu5 by testing these drugs in mGlu5 KO mice bred on a C57BL/6 background (Lu et al., 1997). Pretreatment with fenobam (30 mg/kg i.p.) had no demonstrated effects on mGlu5 KO mice when compared to vehicle treated animals. Of note, the second phase of the formalin test was reduced in vehicle treated KO mice (Figure 7F) to the same extent as in fenobam treated WT mice (Figure 7C). This effect is consistent with the importance of mGlu5 in this phase of the formalin test such that pharmacologic and genetic knockout of mGlu5 both reduce pain related behavior to a similar extent (Chapter 2 Figure 5). The fact that fenobam is without effect in mGlu5 KO mice when compared to vehicle treated littermate mGlu5 KO controls is compelling evidence that fenobam is on target for mGlu5. MPEP (30 mg/kg), in contrast, was found to have robust, residual analgesic effects in mGlu5 KO mice. Questions regarding the selectivity of MPEP for mGlu5 have been noted before (Lea and Faden, 2006), and I report here definitive evidence that the in vivo analgesic effects of MPEP in an

inflammatory pain condition are not exclusively mediated by antagonism of mGlu5. This finding has specific and immediate implications for the pain field, as MPEP has been the primary antagonist used in assessing the role of mGlu5 in pain for over a decade (Lea and Faden, 2006). Previous results obtained by a multitude of research groups, must be considered in light of this finding. However, it is important to note that the data obtained with MPEP in the mGlu5 KO mice should not necessarily be taken to diminish the suggested role for mGlu5 in inflammatory pain. Indeed, as the present study indicates, fenobam is robustly analgesic in inflammatory pain in WT but not mGlu5 KO mice, suggesting its analgesic effects are selective for mGlu5. In addition, as reported in Chapter 2, mglu5 KO mice have reduced hypersensitivity and nocifensive behavior in several animal pain models. Rather than detract from a role for mGlu5 in analgesia, these data suggest an additional mechanism for MPEP in analgesia beyond mGlu5 antagonism.

A major limitation of some analgesics, such as opiates, is that tolerance to their analgesic effects can occur over time. Tolerance to repeated daily dosing of the mGlu5 antagonist MTEP has been demonstrated to occur in rats in the formalin test (Sevostianova and Danysz, 2006). To determine whether tolerance to the analgesic effects of fenobam develops, I injected mice daily for 5 days with an analgesic dose of fenobam (30 mg/kg) or vehicle and then on the 6th day injected mice of both groups with either fenobam or vehicle and then performed a formalin test. Mice chronically injected with fenobam did not display tolerance to the analgesic effects of acute fenobam on the test day. Fenobam retained its analgesic efficacy in both the first and second phase of the

formalin test. It should be noted that this is not conclusive evidence that tolerance to fenobam would not develop if the drug was administered over a longer time course. However it is encouraging preliminary evidence that tolerance may not be a limiting factor in the use of fenobam as an analgesic.

In conclusion, in this chapter I report that the clinically validated mGlu5 antagonist fenobam displays robust analgesic efficacy in three mouse models of inflammatory pain in a manner similar to the prototypical mGlu5 antagonist MPEP. In addition, I demonstrate that the in vivo analgesic effects of fenobam, but not MPEP, are selective for mGlu5. These findings, along with the findings from mGlu5 KO mice reported in chapter 2, and previous reports published in the literature, support a role for mGlu5 antagonists as analgesics. In addition, due to previous reports that fenobam has a good safety profile without oversedation, muscle relaxation, and interaction with ethanol, these data suggest that fenobam may represent a reasonable candidate molecule for testing the analgesic efficacy of pharmacologic blockade of mGlu5 in human subjects. Prior to testing fenobam in human subjects it would be important to explore its potential to cause undesirable side effects. While limited studies in human volunteers (Berry-Kravis et al., 2009), do not report any significant adverse effects of fenobam, in Chapter 4 I present expanded evidence regarding the role mGlu5 may play in locomotion, motor coordination, and appetite by comparing the behaviors of mGlu5 KO mice compared to WT littermates and by testing the effects of fenobam on locomotion, coordination, and appetite in both WT and mGlu5 KO mice.

Chapter 4

The role of mGlu5 in locomotion, motor coordination, and appetitive behavior.

INTRODUCTION

Evidence from both mGlu5 KO mice presented in Chapter 2 and from the mGlu5 antagonist fenobam presented in Chapter 3 suggest a clear role for mGlu5 in nociception. Activation of mGlu5 with the agonist DHPG is pro-algesic, while genetic deletion of mGlu5 and antagonism with fenobam result in decreased nociceptive behaviors. These data provide encouraging evidence that mGlu5 may represent a viable therapeutic target for the treatment of pain. However, any future development of mGlu5 antagonists as analgesics in human patients will require drugs that are both selective for mGlu5 and devoid of significant adverse side effects. Adverse effects may manifest as toxicity of the parent compound or its metabolites. Unwanted effects may also result from antagonism of mGlu5 at sites outside the pain neuraxis. The former could theoretically be solved through medicinal chemistry and the design of new, non-toxic compounds. However deleterious effects that are due to mGlu5 antagonism at sites outside of the pain neuraxis would be impossible to avoid. For this reason I sought to assess both mGlu5 KO mice and fenobam for adverse effects that could prevent the development of mGlu5 antagonists as analgesics.

Additionally, many of the measurements used to assess pain-related behavior rely on intact motor function. Licking, lifting, flinching, and withdrawal from stimuli all require normal motor responses. In Chapters 2 and 3 mGlu5 KO mice and WT mice treated with fenobam were found to have decreases in hypersensitivity following CFA injection and during the formalin test. However it is critical to verify that confounding

alterations in motor coordination or locomotor behavior are not the reason for the perceived reductions in pain-related behaviors. Furthermore, sedation is a common dose limiting factor for some classes of analgesic drugs such as opiates (Melzack and Wall, 2003, p. 389). It would therefore also be warranted to test for potential undesirable sedative effects of mGlu5 antagonists. In this chapter I describe the effects of both fenobam and genetic deletion of mGlu5 on several sensory-motor, locomotive, and coordination tasks. Neither pharmacological inhibition nor genetic deletion of mGlu5 was found to impair motor coordination. However, locomotive behaviors were *increased* in both mGlu5 KO mice and following the administration of fenobam.

Finally, mGlu5 has been previously shown to play a role in appetite and energy balance (Bradbury et al., 2005) and mGlu5 KO mice have also been reported to weigh less than their WT littermates (Bradbury et al., 2005; Xu et al., 2009). I sought to confirm the role of mGlu5 in weight gain and to determine if fenobam mediates any effects on appetitive behavior.

METHODS

Animals: Experiments were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Animal Care and Use Committee of Washington University School of Medicine. Male Swiss-Webster mice (6 to 8 weeks old) were purchased from Taconic. For experiments involving mice lacking mGlu5 (mGlu5 KO; 6 to 8 weeks old), animals were bred inhouse on a C57BL/6 background and compared to WT littermates (Lu et al., 1997). For experiments involving KO animals the experimenter was blinded to genotype. Blinding to genotype was accomplished by using coded ear tag identification numbers and only breaking the code at the end of the experiment. Genotyping of mice bred inhouse was performed using standard PCR techniques as described in Chapter 2. All mice were group housed on a 12/12-light/dark schedule with *ad libitum* access to food and water.

Chemicals and Reagents: Fenobam were purchased from Tocris (Ellisville, MO) and dissolved in DMSO (100%) (Sigma-Aldrich, St. Louis, MO) on the day of experiment. All intraperitoneal (i.p.) injection volumes were 20 microliters. Throughout all experiments the investigator was blinded to pharmacological treatment.

General behavioral testing conditions: Behavioral tests were conducted in the same general manner as described in Chapter 2.

Sensory-Motor Battery: A series of 5 tests was performed on mGlu5 KO mice and their WT littermates to assess gross motor behavior and coordination. All tests were performed 2 times.

Walking Initiation Test: Mice were individually placed in the center of a square outlined in white autoclave tape (21 x 21 cm) on a smooth black surface on a large table top. The time each mouse took to leave the square (place all four paws outside of the tape) was recorded. The maximum time allowed was 60 seconds.

Ledge Crossing Task: Each mouse was tested to see how long it could maintain its balance on a 0.75 cm wide plexiglass ledge without falling (60 seconds maximum). A score of 60 seconds was also assigned if the mouse traversed the entire 51 cm length of the ledge and returned to the starting point in less than 60 seconds without falling.

Vertical Pole Descent Task: Mice were placed head upwards at the top of a vertical metal rod (8 mm diameter, 55 cm height). The rod was finely textured with a file to provide a gripping surface. Mice were given a maximum of 120 seconds to turn 180 decrees and climb down to reach the bottom of the pole and place all four paws on the tabletop. Mice were required to reverse direction and actively climb down. If a mouse slid down the pole without reversing direction or fell down the pole it was given a score of 120 seconds.

Platform Sit: Mice were timed for how long they could remain on an elevated circular platform (1 cm thick, 3 cm in diameter, 47 cm above the tabletop). A maximum score of 60 seconds was assigned to mice that remained on the platform for that amount of time. Mice that climbed down from the elevated platform they were replaced on the platform. If they climbed down a second time they were given a score of 60 seconds. The timer was stopped if a mouse fell.

Screen (Angled and Inverted): Mice were placed on a wire mesh screen (16 squares per 10 cm, 47 cm high x 18 cm wide) set to three different inclinations, 60, 90, and 180 (inverted) degrees. For the 60 and 90 degree inclined tests, each mouse was placed in the middle of the screen in a head up position and timed for how long it required to climb to the top or bottom of the screen. A maximum time of 60 seconds was allowed. For the inverted screen test, mice were placed on the screen oriented at 60 degrees, and then the screen was immediately inverted to 180 degrees. The time mice spent hanging on the screen was measured. A maximum score of 120 seconds was given to an animal that did not fall.

Accelerating Rotarod: An accelerating rotarod (Ugo Basile, Italy) was used to assess motor coordination. For experiments involving mGlu5 KOs, naive WT and KO littermates were given two training sessions separated by one hour. The first training session consisted of two trials of 120 seconds spent walking on the rotarod at a fixed

speed of 4 RPMs. The second training session consisted of one trial of 120 seconds at 4 RPMs. All mice completed the first training session without falling in five attempts or less; all mice completed the second training session in two attempts or less without a fall. Latency to fall as the rotarod accelerated from 4 RPMs to 40 RPMs over 5 minutes was assessed one hour after the second training session. Five consecutive acceleration trials were performed with 10 minutes between each trial.

For experiments designed to assess the effects of fenobam on performance on the accelerating rotarod, naive Swiss Webster mice received the same training paradigm as described above. One hour after the second training session mice were injected i.p. with fenobam (3, 10, or 30 mg/kg), pentobarbital (25 mg/kg) or vehicle (DMS). Latency to fall as the rotarod accelerated from 4 RPMs to 40 RPMs over 5 minutes was assessed 30 minutes post injection. Again, five consecutive acceleration trials were performed with 10 minutes between each trial.

Open Field Locomotor Test: Locomotor activity was measured in an open field using a VersaMax Animal Activity Monitoring System (AccuScan Instruments, Columbus, OH). For experiments involving Swiss Webster mice, animals were habituated to the test room individually in Plexiglas boxes (5 x 5 x 10 inches) for 2 hours. Thirty minutes prior to the assessment of locomotor activity mice were injected i.p. with fenobam (3, 10, or 30 mg/kg) or vehicle and returned to their habituation chamber. Locomotor activity was assessed by recording photobeam breaks in a 42L x 42W x 30H cm chamber for 60 minutes. Total

distance traveled, time spent moving, and the number of beam breaks (horizontal activity), were calculated for the entire chamber, as well as a perimeter (outer 8 cm ring) and center (inner 26 x 26 cm square) region. The percent of time spent in the perimeter was also calculated as (minutes spent in perimeter / 60 minutes x 100%).

For experiments involving naive C57BL/6 WT and mGlu5 KO mice, animals were acclimated to the testing room in their home cage for at least 2 hours. Locomotor activity was measured individually as described above and total distance traveled was calculated for the entire chamber.

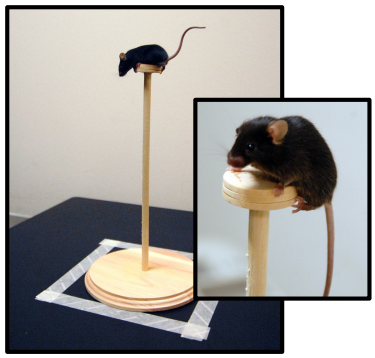
For experiments designed to assess the effects of fenobam on locomotor behavior in mGlu5 KO mice, animals were habituated in their home cages for at least 2 hours, and then injected i.p. with either 30 mg/kg fenobam or vehicle (DMSO) immediately prior to placement in the chamber. Animals were allowed to explore the chamber for 90 minutes and locomotor activity was measured individually as described above and the total distance traveled was calculated for the entire chamber.

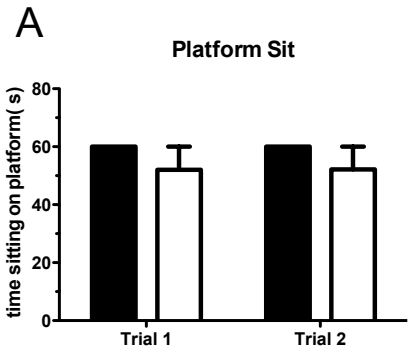
Effects of Fenobam on Food Deprivation-Induced Food Intake: Food deprivation studies were performed in a manner similar to that previously described (Bradbury et al., 2005). For experiments designed to assess the acute effects of fenobam on food intake mice were weighed and housed individually in their home cages three days prior to food deprivation. The bedding was replaced with a wire mesh. On the third day mice were weighed again and food deprived for 24 hours starting at 1 hour after lights on until 1

hour after lights on the next day. Mice were then weighed again and injected i.p. with either 30 mg/kg fenobam or vehicle and pre-weighed food was placed onto the cage bottom. Food intake was measured for each mouse at 15, 30, 60, 90, and 180 minutes, as well as at 3 hours prior to lights off (8 hours later). In order to compensate for variation of body weight, mouse food intake was normalized to body weight. Mice were weighed to the nearest 0.1 g and food was weighed to the nearest 0.01 g.

RESULTS

Effects of mGlu5 Deletion on Sensory-Motor Behavior: No differences between WT and mGlu5 KO littermates were noted when mice were required to sit on a platform for 60 seconds (**Figure 1a**), climb down from a pole (**Figure 1b**), initiate movement after being placed in an open space (**Figure 1f**), or walk across a ledge (**Figure 1g**). No differences were found between the time WT and mGlu5 KO mice took to reach the edge of a 90 degree screen (**Figure 1d**), or hang inverted from the same screen (**Figure 1e**), however there was a difference when mice were required to reach the edge of a 60 degree screen (**Figure 1c**).





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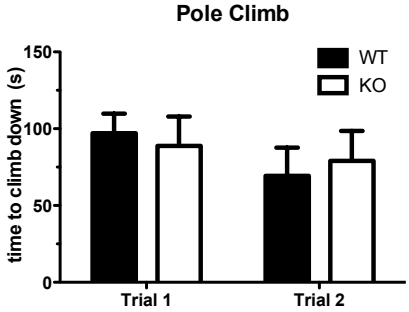

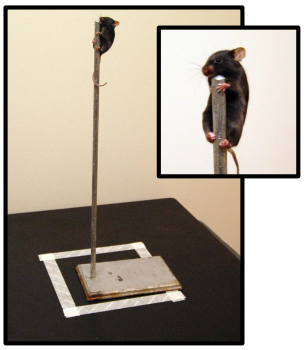

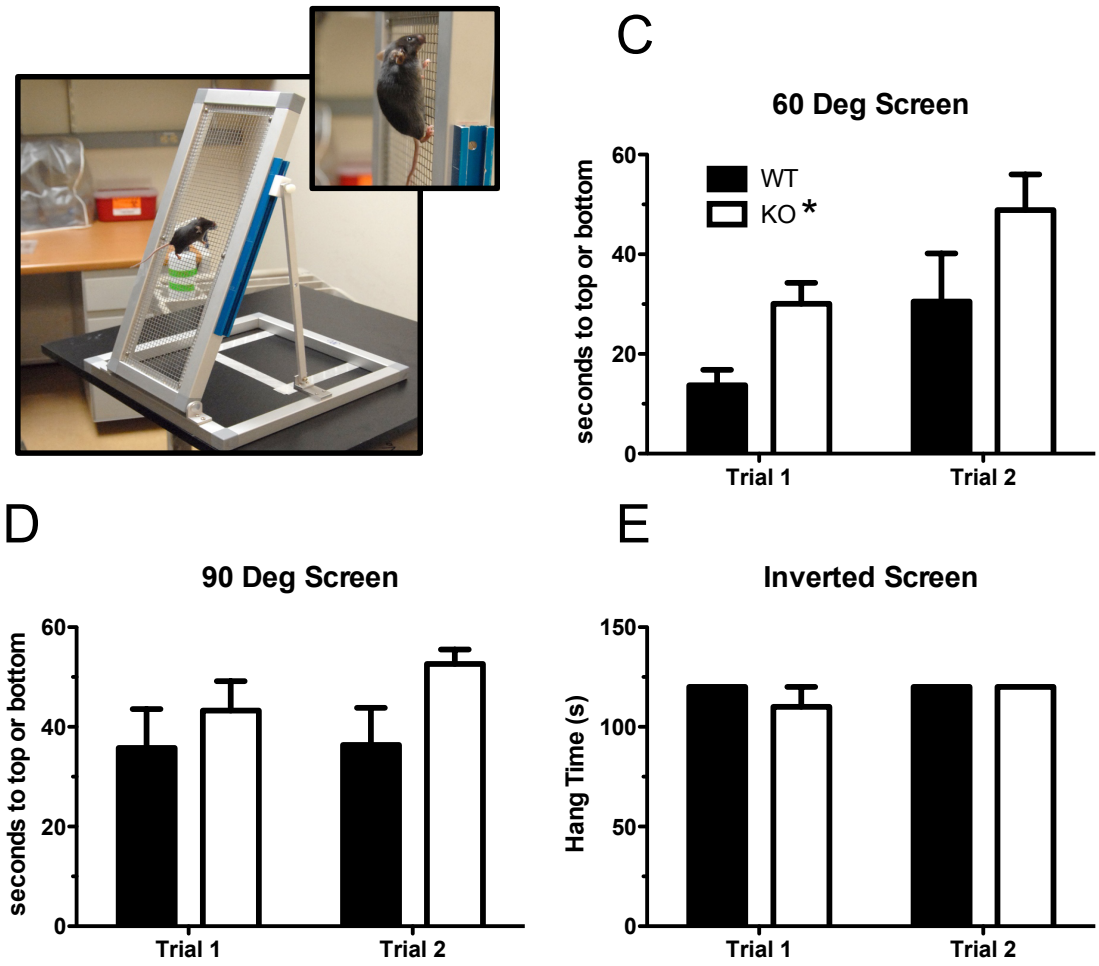

Figure 1: The effects of mGlu5 Deletion on Sensory-Motor Behavior

mGlu5 and WT littermate mice had no differences in platform sit or pole climb (a-b, on previous page; p=0.17, 0.97 respectively), (d) time to reach the edge of a 90 degree screen (p=.07), or (e) time hanging from an inverted screen (0.33). mGlu5 KO mice took significantly longer to reach the edge or bottom of a 60 degree screen (c, 2-Way ANOVA Main Effect of Fenobam p = 0.025; No differences on Bonferroni Post-hoc test). All unlabeled statistics are presented as 2-Way ANOVA Main Effect of Genotype. n = 7 per group.

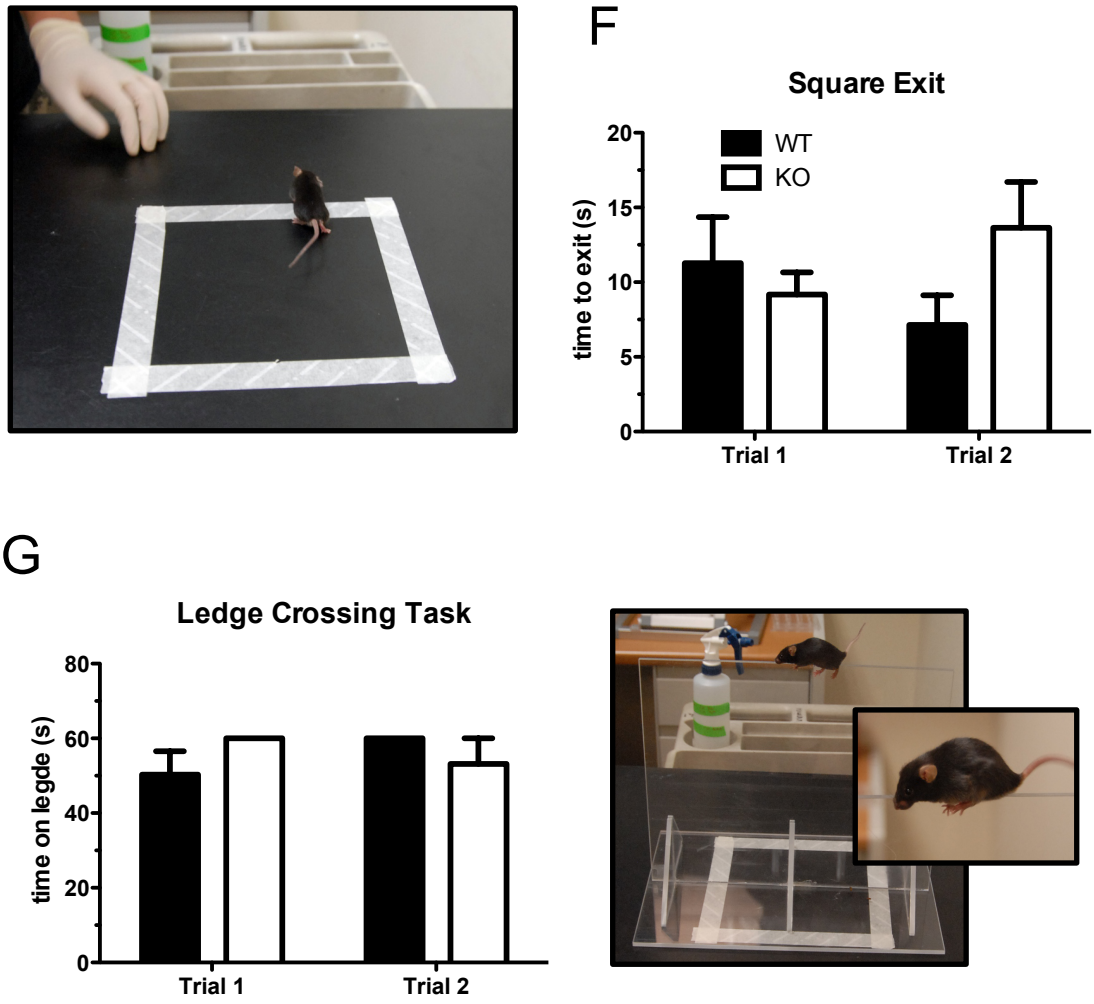

Figure 1: The effects of mGlu5 Deletion on Sensory-Motor Behavior (continued)

mGlu5 and WT littermate mice exhibited no differences in (f) square exit or (g) ledge crossing (p=0.39, 0.76 2-Way ANOVA Main Effect of Genotype respectively). n = 7 per group.

Neither mGlu5 Deletion nor Pharmacological Inhibition Affects Performance on the Accelerating Rotarod: mGlu5 KO mice and their littermate controls showed no differences on the accelerating rotarod (4 – 40 rpm increase over 5 min over five consecutive trials) (**Figure 2a**). As compared to vehicle, fenobam injection resulted in no significant differences in latency to fall from an accelerating rotarod at any dose tested, up to 30 mg/kg (**Figure 2b**). No significant difference between vehicle-injected and fenobam-injected Swiss Webster mice was observed over five consecutive trials. In order to demonstrate that a reduction in fall latency could be induced, one group of mice was injected with 25 mg/kg of pentobarbital, which resulted in a significant reduction in the fall latency in both the first and second trials, when compared to vehicle. All groups of Swiss Webster mice showed significant improvement in fall latency from the first to the fifth trial.

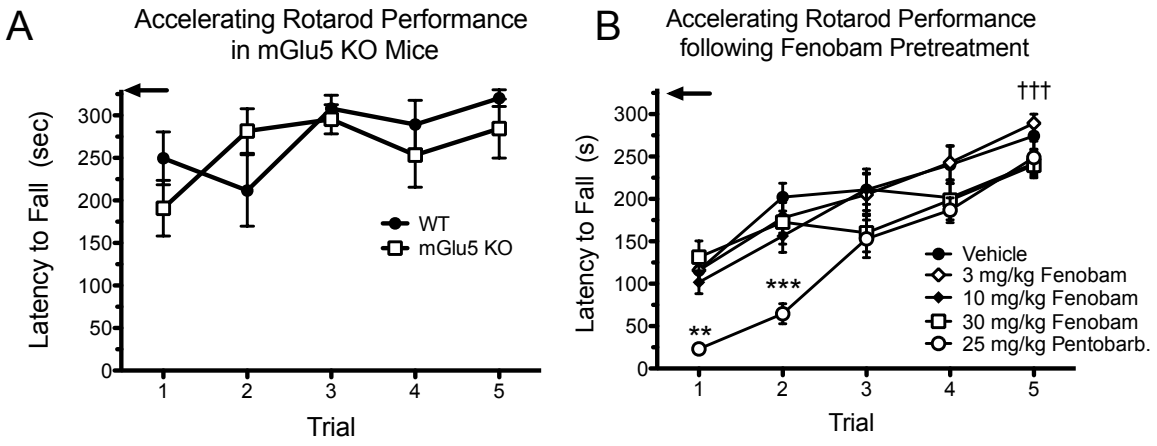

Figure 2: The effects of mGlu5 Deletion on Accelerating Rotarod Performance

(a) mGlu5 and WT littermate mice had no differences in performance on the accelerating rotarod (2-Way ANOVA Main Effect of Genotype p=0.65, n=7 per group). (b) Fenobam administered 30 min prior to placement on the accelerating rotarod did not affect latency to fall at any dose tested when compared to vehicle. Pentobarbitol significantly decreased the latency to fall on both the 1st and 2nd trial (2-Way ANOVA Main Effect of Drug $p<0.0001$, Bonferonni Post Test **, *** = $p<0.05$, 0.01). All groups showed significant improvement from the 1st to 5th trial (2-Way ANOVA Main Effect of Time $p<0.0001$, Bonferroni Post Test ††† = $p<0.001$). n = 10-12 per group.

Both mGlu5 Deletion and Fenobam Increase Locomotor Activity in the Open Field Task: The open field task was used to assess the effects of genetic deletion and pharmacological inhibition of mGlu5 on locomotor activity. Swiss Webster mice were pretreated with fenobam (3, 10, and 30 mg/kg i.p.) 30 min. prior to placement in the field and their locomotor activity was measured. As compared to vehicle-treated animals fenobam produced no significant effect on spontaneous exploratory behavior at 3 and 10 mg/kg, however at 30 mg/kg a significant increase in exploratory behavior was observed in the total distance mice traveled over 60 minutes (**Figure 3a,b**). No significant effects on behaviors in the center (26 cm x 26 cm) of the open field apparatus were observed between the different doses (data not shown). No dose of fenobam was found to alter the percent of time mice spent in the perimeter versus the center (**Figure 3c,d**).

When the effects of genetic deletion of mGlu5 were examined in the open field it was found that drug naive mGlu5 KO mice traveled significantly farther in the open field over a 60 minute time period as compared to their drug naive WT littermates (**Figure 3e,f**).

To assess whether the increased locomotor activity following fenobam administration was due to inhibition of mGlu5, KO mice and WT littermates were injected with fenobam (30 mg/kg) or vehicle (DMSO) and then immediately placed in the open field for 90 minutes. There was a significant increase in the total distance travelled in the fenobam treated WT mice and both the vehicle and fenobam treated mGlu5 KO as compared to vehicle treated WT mice (**Figure 3g,h**).

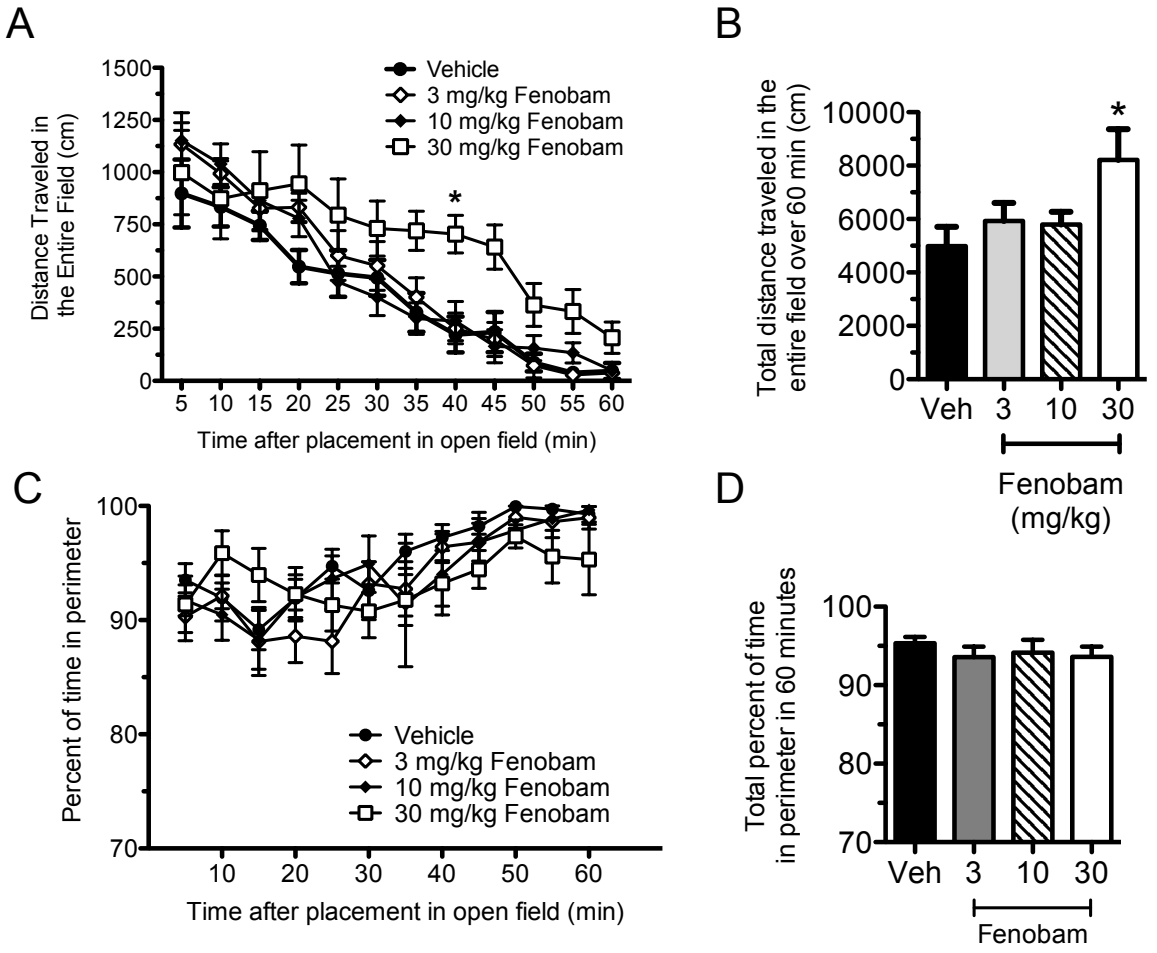

Figure 3: The effects of fenobam and mGlu5 Deletion on Open Field Locomotor Behavior

When administered 30 min prior to placement in a novel open field environment, Fenobam (30 mg/kg, i.p.) significantly increased the total distance mice traveled as compared to vehicle (a) between 35 and 40 min (2-Way ANOVA Main Effect $p<0.0001$; Bonferroni Post Test $p<0.05$) and (b) as a sum total of distance traveled in 60 min (1-Way ANOVA $p=0.0393$, Dunnett's Post Test $p<0.05$) (c, d) Fenobam did not decrease the amount of time mice spent in the perimeter (outer 8cm ring) as compared to the center (inner 26 x 26 cm square) at any dose tested (2-Way ANOVA Main Effect of Fenobam $p=0.11$). (n = 10-11 per group.)

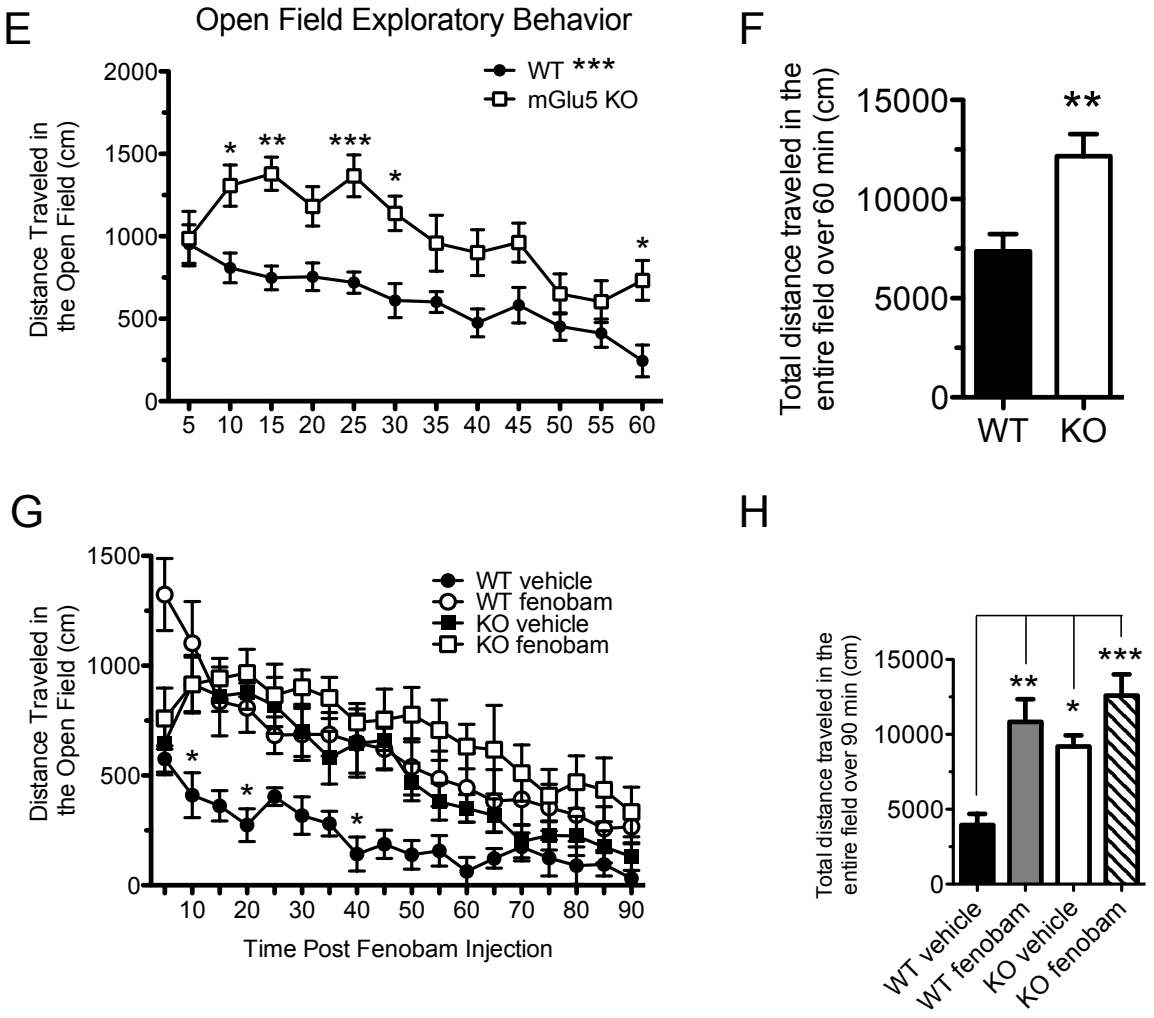

Figure 3: The effects of fenobam and mGlu5 Deletion on Open Field Locomotor Behavior (continued)

Drug-naive mGlu5 KO mice traveled significantly farther compared to their WT littermates at multiple time points (e, 2-Way ANOVA Main Effect $p<0.0001$; Bonferroni Post Test *,**,*** $p<0.05$, 0.01, 0.001) and as a sum total of distance traveled in 60 min (f, Unpaired t-test $p=0.0044$) n = 8 per group. (g-h) WT mice traveled significantly less than all other groups at multiple time points (g, 2-Way ANOVA Main Effect $p<0.0001$; Bonferroni Post Test * $p<0.05$) and as a sum total of distance traveled in 90 min (h, 1-Way ANOVA $p=0.0001$, Bonferroni Post Test *,**,*** $p<0.05$, 0.01, 0.001) Fenobam did not effect the total distance traveled in mGlu5 KO mice as compared to vehicle treated mGlu5 KOs (Bonferroni Post Test $p>0.05$). n = 7-8 per group.

mGlu5 is Required for Normal Weight Gain and Feeding Behavior: Age matched mGlu5 KO mice weigh significantly less than their WT littermates (**Figure 4a,b**). The effects of fenobam on food intake following a 24 hour food deprivation was also assessed in WT Swiss Webster mice. Fenobam (30 mg/kg) administered at the time of refeeding significantly decreases the amount of food consumed, as compared to vehicle treated animals. No differences in body weight were noted in animals prior to re-feeding (Veh = 32 +/- 0.82 g, Fenobam = 31.73 +/- 1.25 g; unpaired T-test p=0.86). At 8 hours food intake of the fenobam treated mice was not different from vehicle treated animals (Veh = 97.3 +/- 8.8 mg/g; Fenobam = 87.9 +/- 7.8 mg/g; unpaired T-test p=0.44).

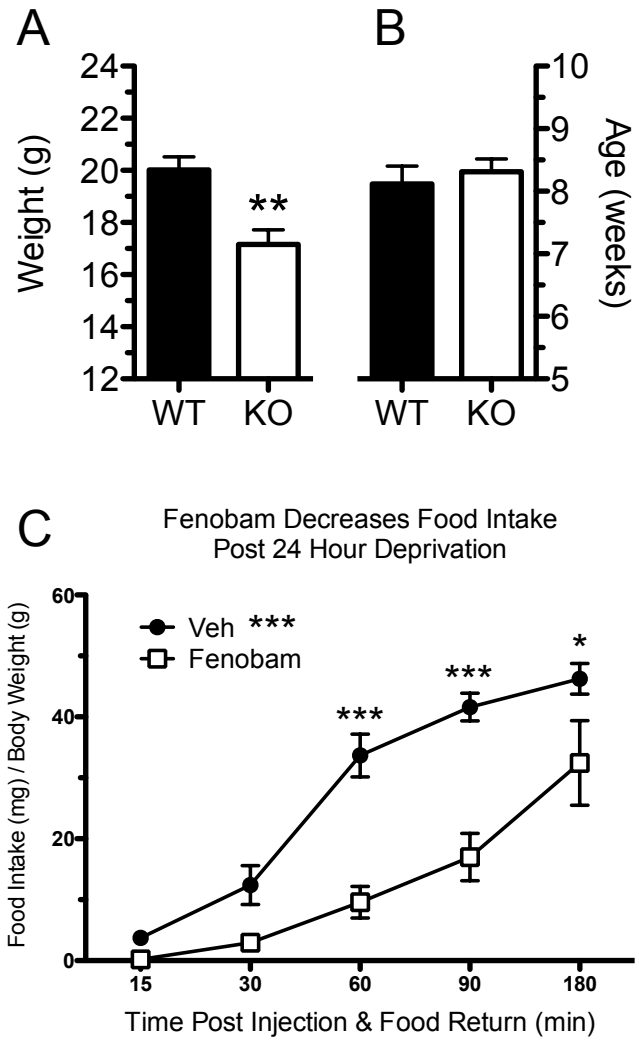

Figure 4: The effects of mGlu5 Absence and Inhibition on weight gain and post-fasting food intake.

(a) mGlu5 KO mice weigh significantly less than their WT littermates (Unpaired t-test p=0.0012). (b) No differences in the age of animals was noted (Unpaired t-test p=0.58). n = 10 - 11 per group. (c) Fenobam (30 mg/kg) significantly decreased food intake following a 24 hour fast as compared to vehicle (2-Way ANOVA Main Effect of Fenobam $p<0.0001$, Bonferroni Post Test *, *** = $p<0.05$, 0.001). n = 6 per group.

DISCUSSION

An assessment of motor coordination, locomotive behavior, and appetitive behavior was performed in mGlu5 KO mice and WT mice treated with the mGlu5 antagonist fenobam. No differences were seen in mGlu5 KO as compared to their WT littermates in any sensory motor behavior task with the exception of a significant reduction in the time to reach the edge of a 60 degree screen. Interestingly mGlu5 KO mice did not exhibit any differences from WT controls on a 90 degree screen. The biological significance of a reduction in time to reach the edge of a 60 degree when all other tests of sensory motor function are normal is questionable, especially when there is no difference when that very same screen is oriented to 90 degrees inclination. Perhaps more importantly, there were no differences in tests that are viewed as significantly more challenging, including climbing across a narrow ledge, and down from a narrow pole. Both of these two tasks require a mouse to navigate with limited foot purchase and execute complex turning behaviors without falling. A test of strength where mice were required to hang upside down for two minutes also yielded no differences.

Furthermore, no differences between mGlu5 KO and WT mice were seen on another test of motor coordination, the accelerating rotarod. Fenobam treated mice also exhibited no differences compared to vehicle treated controls on the accelerating rotarod. These findings are interesting in light of findings from previous studies of MPEP and the related mGlu5 antagonist MTEP that have reported undesirable impairment of locomotion and motor coordination at analgesic doses (Zhu et al., 2004). While the

results presented in Chapter 4 suggest that mGlu5 is not in fact required for normal motor coordination, administration of fenobam at the analgesic dose (30 mg/kg) but not at lower doses (3 and 10 mg/kg) resulted in an increase in spontaneous locomotor activity in the open field assay.

This increase in locomotion is in contrast to the decreased locomotion reported with MPEP (Zhu et al, 2004). Previous clinical trials of fenobam in human patients (Friedmann et al., 1980) have reported that fenobam may have psychostimulant properties. It is possible that the observed increases in locomotor activity reported here are due to a similar psychostimulant effect that is separate from the locomotor inhibition seen with MPEP. It should be noted that in figure 3a and 3b fenobam is administered 30 minutes prior to beginning open field testing and that the increases in locomotor activity as compared to vehicle do not become readily apparent until the 40 minute time point (70 minutes post drug injection). Based on findings from our drug disposition studies (Chapter 3), fenobam clearance is nearly complete after approximately one hour and increased locomotion is only seen *after* the drug is likely cleared. Therefore, it is possible, however speculative, that the observed increase in locomotor activity is due to the accumulation of fenobam metabolites and not the parent compound itself. Alternatively this could represent a "rebound" effect of unblocking mGlu5.

However, as seen in Figure 3e-h, mGlu5 KO mice exhibited increased locomotor activity at multiple time points when compared to WT littermates. In addition when fenobam injection occurs immediately prior to placement in the open field increased

locomotor activity is also exhibited at multiple times points. These two findings suggest that the increased locomotor activity is likely due to antagonism of mGlu5. Findings that fenobam does not increase locomotor activity in mGlu5 KO mice also suggest that the effect is mediated by mGlu5, although it is possible that ceiling effects are preventing any increased locomotion in mGlu5 KO mice.

As mentioned, these findings of increased locomotor activity are in opposition to those seen with MPEP (Spooren et al., 2000; Zhu et al., 2004) and the related mGlu5 antagonist MTEP (Zhu et al., 2004), which have both been shown to reduce locomotor activity at analgesic doses. As the dose of fenobam that resulted in changes in locomotor activity is the same as that which reduced spontaneous formalin behavior (Chapter 3), it is possible that the analgesic effects of fenobam will prove inseparable from certain other effects. However, motor coordination as measured on an accelerating rotarod was not affected by fenobam at doses up to 30 mg/kg. Thus, while fenobam may increase locomotor activity, the potentially more deleterious side effect of altered motor coordination appears to be absent at the tested analgesic dose. Furthermore the stimulant side effects of fenobam could actually be beneficially exploited in patients that are also suffering from concurrent depression or malaise, assuming that any psychostimulant effects are not aversive. No significant adverse reactions were reported when three healthy volunteers (Berry-Kravis et al., 2009) where administered fenobam at doses of 150 mg, so it is hoped that this will be the case. Only further clinical testing in humans will tell.

A fascinating effect of mGlu5 deletion and pharmacological inhibition that is likely unrelated to analgesia is a significant effect on weight gain and post-fast feeding behavior. mGlu5 KO mice have been previously reported to weigh less than their WT littermates (Bradbury, et al., 2005; Xu et al., 2009) and I confirm this finding here. Differences in *ad libitum* food intake were not found (Bradbury, et al. 2005), so this is not believed to account for the differences. However both mGlu5 KO mice and mice injected with MPEP exhibit significantly less re-feeding behavior following a 14-hour fast, suggesting that mGlu5 does play a role in appetite and feeding behavior (Bradbury et al., 2005). Here I report that fenobam administration also significantly decreases post-fast re-feeding. Fenobam's psychostimulant properties and appetite suppressive effects should certainly be viewed as separate from its analgesic effects. However, rather than being dose limiting, it might be possible to exploit these effects to beneficial clinical use in certain diseases that have pain as a major symptom. Osteoarthritis is a prime candidate as weight loss has been associated with both a decrease in risk of developing osteoarthritis (Felson et al, 1997) and both weight loss and increased activity result in an improvement of existing osteoarthritis symptoms (Martin et al., 2001). Again, further testing in human patients will be necessary to know for certain.

Chapter 5

The Pain Related Behaviors of Peripherally Restricted Conditional mGlu5 KO Mice

INTRODUCTION

The findings presented above strongly suggest that mGlu5 represents a viable target for the treatment of pain. However, as presented in chapter 4, mice globally deficient in mGlu5 and WT mice treated systemically with the mGlu5 antagonist fenobam both exhibit altered behaviors unrelated to analgesia including increased locomotor activity and altered weight gain and feeding behavior. While I make the case that these effects may be of beneficial clinical utility in some patients, it is also possible that they will be detrimental. As mGlu5 is expressed widely throughout the brain, it is also possible that other centrally mediated effects of systemically active mGlu5 antagonists will also be detrimental. However, if mGlu5 expressed on peripheral nociceptive neurons was found to play a significant role in pain transmission then it might be possible to develop non-brain penetrant, peripherally restricted mGlu5 antagonists that would be both analgesic and devoid of centrally mediated adverse effects.

While glutamate is the predominant excitatory neurotransmitter in the CNS it has also been demonstrated to play a significant role as an inflammatory mediator in peripheral tissues. Metabotropic glutamate receptor 5 is expressed on the peripheral terminals of unmyelinated nociceptive neurons in the skin (Bhave et al., 2001). In addition, the concentration of glutamate is increased in the skin following formalin injection into the hind paw of the rat (Omote et al., 1998), and in the synovial fluid of arthritis patients (McNearney et al, 2000), suggesting that peripherally released glutamate

may be able to activate peripheral mGlu5 in pain states. As demonstrated in chapter 3, peripheral injection of the group I mGluR agonist DHPG into the mouse hindpaw is sufficient to generate thermal hyperalgesia that is absent in mGlu5 KO mice. Findings that peripherally administered MPEP is analgesic in both DHPG-induced hypersensitivity and in the formalin test (Bhave et al., 2001; Walker et al., 2001b) support the idea that peripherally expressed mGlu5 could be targeted to analgesic effect. However, the findings presented in chapter 3 that MPEP retains analgesic activity in mGlu5 KO mice suggests that it may be exerting additional analgesic effect through an mGlu5-independent mechanism. In this chapter I sought to address the question of whether peripheral mGlu5 is required for the full expression of pain by employing a genetic approach. I generated a line of conditional knockout mice lacking mGlu5 only in primary-afferent nociceptors. I assessed the DHPG- and formalin-induced pain related behaviors of these mice, as well as their performances on sensory-motor tasks.

These Nociceptor Specific mGlu5 (NSmGlu5) conditional knockout mice were generated by using a Cre-LoxP technique. Mice in which the seventh coding exon was flanked by loxP (floxed mGlu5) were crossed with a Cre-expressing transgenic mouse line (SNS-Cre) in which Cre-Recombinase is expressed only in small diameter DRG neurons that express Sensory Neuron Specific Sodium Channel NaV1.8. Nav1.8 is selectively expressed in the subset of sensory neurons which are C-fiber nociceptors (Akopian et al., 1996). The resulting offspring from these crosses will only lack mGlu5 in peripheral sensory neurons that express NaV1.8, including virtually all unmyelinated

neurons. (Agarwal et al., 2004). Initially there were technical hurdles involving Cre-mediated recombination of mGlu5 in tissues outside of the peripheral nervous system. However, I report here that NSmGlu5 mice do not differ from floxed mGlu5 littermates in two different pain tests, suggesting a less prominent role for peripheral mGlu5 in pain than was previously suggested from studies using mGlu5 antagonists.

METHODS

NSmGlu5 Breeding Scheme: Experiments were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Animal Care and Use Committee of Washington University School of Medicine. Nociceptor specific conditional mGlu5 KO (NSmGlu5) mice (6 to 8 weeks old) were bred inhouse on a C57BL/6 background and compared to floxed mGlu5 littermates. NSmGlu5 mice were generated by crossing floxed mGlu5 mice with a Cre-expressing transgenic mouse line, in which Cre is only expressed in small diameter DRG neurons that express Sensory Neuron Specific Sodium Channel $Na_V1.8$ (SNS-Cre). Floxed mGlu5 mice were generated and provided by Jian Xu and Steve Heinemann (Xu et al., 2009). We used mice in which the Neo-cassette has been floxed out. SNS-Cre mice were generated and provided by Nitin Agarwal and Rohini Kuner (Agarwal et al., 2004).

NSmGlu5 mice were generated by crossing the floxed mGlu5 mice with the SNS-Cre mice through a series of three crosses: In Cross 1: SNS-Cre mice were crossed to floxed mGlu5 mice, generating 50% offspring that are heterozygous for both the Cre and the floxed allele (Cre/+ \\ Flox/+). In Cross 2: Cre/+ \\ Flox/+ mice were crossed to floxed mice generating 25% offspring that are NSmGlu5 (Cre/+ \\ Flox/Flox). In Cross 3: These NSmGlu5 mice were mated to floxed mice, resulting in 50% floxed mice (+/+ \\ Flox/ Flox) and 50% NSmGlu5 mice (Cre/+ \\ Flox/Flox). Floxed mGlu5 and NSmGlu5 (Cross 3) were used to maintain the colony and produce progeny for experiments. NSmGlu5 mice heterozygous for the $Na_V1.8$-Cre transgene, and homozygous for the floxed mGlu5

allele (Cre/+ \\ FLOXmGlu5/FLOXmGlu5) were compared to control floxed mGlu5 littermates that retain full mGlu5 expression (+/+ \\ FLOXmGlu5/FLOXmGlu5).

Blinding to genotype was accomplished by using coded ear tag identification numbers and only breaking the code at the end of the experiment. Unless otherwise specified only male mice were tested. All mice were group housed on a 12/12-light/dark schedule with lights on at 0600 CST and *ad libitum* access to food and water.

PCR Genotyping: Genotyping was performed using standard PCR techniques. Two different genotyping protocols were used to identify the SNS-Cre transgene and the presence of floxed mGlu5.

SNS-CRE Genotyping: One set of genotyping primers were used to verify the presence of the SNS-Cre transgene.

Primers:

1) Forward Primer: TAT CTC ACG TAC TGA CGG TG

2) Reverse Primer: AGA CTA ATC GCC ATC TTC CAG C

Genotyping Reaction Master Mix (per reaction):

1) MilliQ Water: 19.8 µl

2) GoTaqFlexi (Promega) 5x Buffer: 5 µl

3) $MgCl_2$ (25 mM): 1.5 µl

4) Forward Primer: 0.5 µl

5) Reverse Primer: 0.5 µl

6) dNTPs (10 mM): 0.5 µl

7) GoTaq (Promega): 0.2 µl

8) Extracted Tail DNA - 1 µl

SNS-Cre PCR Protocol:

Cycle 1 (1x): Step 1: 94^{o}C for 5 min

Cycle 2 (40x): Step 1: 94^{o}C for 1 min

Step 2: 60^{o}C for 1 min

Step 3: 72^{o}C for 1 min

Cycle 3 (1x): Step 1: 72^{o}C for 10 min

Hold at 4^{o}C until agarose gel separation.

Gel Separation: PCR product separation on a 1% agarose gel yields a single band of ~500 bp in mice that express the transgene. No band is detected in mice that did not possess the transgene.

Floxed mGlu5 Genotyping: The entire 7th coding exon of mGlu5 was flanked by LoxP as described and two sets of primers were used for detecting the floxed allele.

Primers:

Set A: 1) Forward Primer — 153 — AGA TGT CCC ACT TAC CTG ATG T

2) Reverse Primer — 154 — AGT TCC GTG TCT TTA TTC TTA GC

3) Deletion Primer — 316 — AGG CGC TTC CAA AAT AGA GG

Set B: 1) Neo Primer — 72 — GGC TCT TTA CTA TTG CTT TAT GAT AAT G

2) Forward Primer — 126 — TTG CTA GCT GAA AAG GAC GAA ACA

3) Reverse Primer — 127 — TCG TTT TGA ATC TTG GGG ACA GTT AC

Genotyping Reaction Master Mix (per reaction):

1) MilliQ Water: Primer Set A: 9.4 µl ; Primer Set B: 7.9 µl

2) GoTaqFlexi (Promega) 5x Buffer: 4 µl

3) $MgCl_2$ (25 mM): 2 µl

4) 1st Primer — if 154: 1 µl or if 72: 2.5 µl

5) 2nd Primer — (154 or 126): 1 µl

6) 3rd Primer — (316 or 127): 1 µl

7) dNTPs (10 mM): 0.4 µl

8) GoTaq (Promega): 0.2 µl

9) Extracted Tail DNA - 1 µl

Floxed mGlu5 PCR Protocol (use for both primer sets):

Cycle 1 (1x): Step 1: 94°C for 3 min

Cycle 2 (35x): Step 1: 94°C for 30 seconds

Step 2: 52°C for 30 seconds

Step 3: 72°C for 30 seconds

Cycle 3 (1x): Step 1: 72°C for 2 min

Hold at 4°C until agarose gel separation.

Gel Separation: PCR product separation on a 2% agarose gel yields two different sets of three bands, depending on which primer set was used.

Primer Set A: Primer 153 hybridizes ~50 bp upstream form the first loxP site, primer 154 hybridizes ~300 bp upstream from exon 7, and primer 316 is ~240 bp downstream of the last loxP site. PCR using primer set A yields bands of ~200 bp, ~270 bp and ~320 bp from the *WT*, *floxed mGlu5* (with *neo* deletion) and *del* allele respectively.

Primer Set B: Primer 126 hybridizes ~315 bp downstream of exon 7, primer 127 is ~120 bp downstream of the last loxP site, and the neo primer (72) is within the neo cassette, ~300 bp upstream of the last loxP site. DNA from *WT*, *floxed mGlu5* (with *neo*), and *floxed mGlu5* (without *neo*) yields 217 bp, 463 bp, and 323 bp PCR products respectively.

Reverse Transcription PCR rtPCR: rtPCR experiments required four steps, 1) Tissue harvest and isolation, 2) total RNA purification, 3) Reverse Transcription of tRNA to cDNA, 4) PCR of cDNA

Tissue Isolation: Mice were deeply anesthetized with a 100 µl injection of ketamine, acepromizine, and xylazine (KAX) (42.86 mg/ml, 8.57 mg/ml, and 1.43 mg/ml KAX respectively). Fresh lumbar DRGs were identified by dissecting the sciatic nerve back to the DRG just outside the spinal cord. DRGs were then mechanically isolated using iridectomy scissors, and transferred to RNA Later solution (Applied Biosystems/Ambion, Austin, TX). Hippocampus was isolated from fresh brain, diced into <2 mm^3 pieces with a scalpel and transferred to RNA later solution. All tissue samples were stored at -20^oC until RNA purificiation.

Total RNA Purification: RNA was recovered from tissue using an RNeasy Micro Purification Kit (Qiagen, Valencia, CA). Tissue was placed into 350 microliters of Buffer RLT (Qiagen proprietary solution) with 1% β-Mercaptoethanol and mechanically homogenized with a VWR Pellet Mixer and disposable RNASE-free plastic pestal (VWR International, Arlington Heights, IL). Tissue was homogenized until naked-eye visible macroscopic pieces were no longer present. The lysate was pipetted directly into a QIAshredder spin column (Qiagen, Valencia, CA) and centrifuged at 18K x g for 2 minutes. Additional steps for RNA purification were carried out according to the Qiagen

RNeasy Micro Handbook (Second Edition, December 2007, pp. 27-29, steps 4 to 13). All pipette tips were RNASE-free.

Reverse Transcription and Genomic DNA Elimination: rtPCR was performed immediately following RNA purification using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Four microliters of total RNA were used as template. The protocol was carried out according to the Qiagen QuantiTect Reverse Transcription Handbook (January 2009 Edition, pp. 12-13). cDNA was stored at -20^{o}C until performing PCR reactions.

PCR Genotyping: Two different primer sets were used, one directed at exon 1, which is deleted in the global mGlu5 KO mice described in Chapter 2, and one directed at exon 7, which is flanked by LoxP in the floxed mGlu5 mice generated by Xu et al. (2009).

Primers:

Set 1: Forward Primer is in Exon 1, Reverse is in Exon 7

Forward Primer 1: GAG GGT TGT ACC TTC GGA TG

Reverse Primer 1: CCA GTA TCT TCG ATG GGG TG

Set 6: Forward Primer is in Exon 6, Reverse is in Exon 8

Forward Primer 6: CTG GCC CAC TGA CGA CTT

Reverse Primer 6: CCC AGA ATG AGA AGA GCA C

Genotyping Reaction Master Mix (per reaction):

1) MilliQ Water: 13.8 µl

2) GoTaqFlexi (Promega) 5x Buffer: 4 µl

3) $MgCl_2$ (25 mM): 2.5 µl

4) Foward Primer: 1 µl

5) Reverse Primer: 1 µl

6) dNTPs (10 mM): 0.5 µl

7) GoTaq (Promega): 0.2 µl

8) cDNA - 1 µl

PCR Protocol (use for both primer sets):

Cycle 1 (1x):	Step 1: 94°C for 4 min
Cycle 2 (35x):	Step 1: 94°C for 45 seconds
	Step 2: 55°C for 45 seconds
	Step 3: 72°C for 45 seconds
Cycle 3 (1x):	Step 1: 72°C for 5 min
	Hold at 4°C until agarose gel separation.

Gel Separation: PCR product separation on a 2% agarose gel yields two different sets of bands, depending on which primer set was used.

Primer Set 1: The forward primer hybridizes within the 0.4 kb portion of exon 1 that has been deleted from the global mGlu5 KO mice. It is approximately 75 base pairs from the exon 1 and 2 junction. The reverse primer hybridizes in exon 7, approximately 20 base pairs from the exon 6 and exon 7 junction. Primer set 1 yields an ~1160 bp product in WT mice and no product in global mGlu5 KO mice derived from John Roder's colony (See Chapter 2).

Primer Set 6: The forward primer hybridizes in Exon 6, approximately 5 bp from the exon 6 and exon 7 junction. The reverse primer hybridizes in Exon 8, approximately 25 bp from the exon 7 and exon 8 junction. Primer set 6 potentially yields two bands, one of ~1100 bp if the floxed exon (exon 7) is present, and one of ~160 bp if the 940 bp floxed exon has been excised.

Reagents and Drugs used in Behavioral Experiments: DHPG and formalin were from the same sources and prepared in the same manner as described in Chapter 2.

General behavioral testing conditions: Behavioral tests were conducted in the same general manner as described in Chapter 2.

Accelerating Rotarod and Open field Locomotor Test: The accelerating rotarod task and open field locomotor tests were performed in the same manner as used to test mice globally deficient in mGlu5 as described in Chapter 4.

Intrathecal DHPG-Induced Spontaneous Behavior: Spontaneous i.t. DHPG-induced behavior was assessed in the same manner as described in Chapter 2.

Spontaneous Formalin-Induced Nocifensive Behavior: Spontaneous formalin induced nocifensive behavior was assessed in the same manner as described in Chapter 2 with one exception. Since spontaneous nocifensive behavior was generally absent from 45 to 60 minutes in previously tested animals, behaviors were scored only for 45 minutes, instead of 60 minutes.

Data Analysis: Statistical analysis of behavioral data was performed in the same manner as described in Chapter 2.

RESULTS

Verification of Peripherally Restricted Deletion of mGlu5

PCR Genotyping: Genotyping from tail DNA was expected to produce bands at ~200 bp and ~270 bp corresponding to WT and floxed mGlu5 genotype mice respectively. However, a third band was also seen at ~320 bp in some mice (**See Figure 1**). This ~320 band corresponds to a genotype where exon 7 of mGlu5 has been excised (Xu et al, 2009). Initially genotyping was performed using a different set of primers (Xu et al, 2009), and as such this extra band was not immediately discovered. PCR of tail DNA taken from several mice that were used as breeders in this colony were later found to produce a band at ~320 bp. Eleven of the first 53 breeders that were set up had a band at ~320 bp. At least one mouse in 9 out 21 matings in the colony (42.9% of all matings) had a band at ~320 bp. The presence of the CRE transgene did not affect whether the band at ~320 bp was seen. Mice B and D had the SNS-Cre transgene, while mice A and C did not (data not shown). Some mice that were CRE-negative were found to have had a band at ~320 bp. Whether the CRE-transgene was present in the mother or the father did not affect the appearance of a band at ~320 bp in offspring.

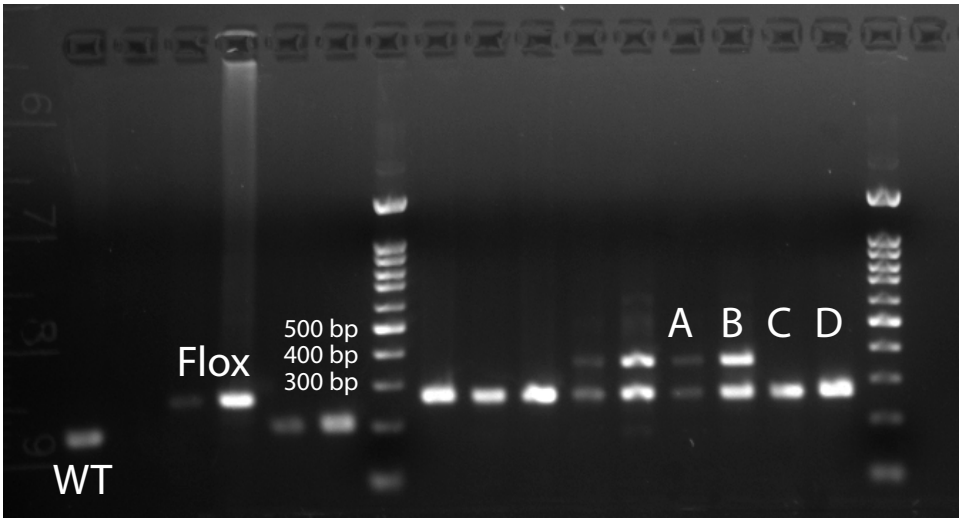

Figure 1: Gel of a PCR from Floxed mGlu5 Genotyping.

A representative gel showing PCR results from genotyping tail DNA from NSmGlu5 mice and their floxed littermates. The bands on the left labelled WT and Flox are control DNA from animals previously genotyped. Note the two bands in lane A and B corresponding to ~270 and ~320 bp respectively. Only the ~270 bp band is present in lanes C and D. Mice from A and B are interpreted as having one copy of the floxed mGlu5 allele and one copy where the floxed section has been deleted to make a global KO allele. Mice in lane C and D are interpreted as being homozygous floxed. Note that mice A and C did not have the SNS-Cre Transgene, while mice B and D did. Thus, mouse A and B are actually heterozygous for mGlu5, while mouse C is a true Flox mGlu5 mouse with no mGlu5 deletion at either locus and mouse D is an NSmGlu5 mouse, homozygous for the floxed mGlu5 and possessing the SNS-Cre transgene.

rtPCR: PCR amplification of cDNA from mouse DRGs and hippocampus was performed to detect the different mGlu5 RNA species present in those tissue types. Two different sets of primers were used to detect deletion of mGlu5. **Figure 2** is a schematic of the exons that make up the mGlu5 cDNA and the locations of the primers in that cDNA. The four animals that are labelled A-D in **Figure 1** were assessed for different cDNA species by rtPCR (**Figure 3**). Animals that had a ~320 bp band in the genotyping PCR (A and B) exhibited two bands when rtPCR was performed with primer set 6, one at ~160 bp and one ~1100 bp. These two bands were seen in both DRGs and hippocampus (**Figure 3**). Animals that did not have the band at ~320 bp (C and D) only had two bands when rtPCR was performed in DRGs (**Figure 3**). When rtPCR was performed on additional mice, including WT and global mGlu5 KO mice, two bands were only seen using primer set 6 in NSmGlu5 mice (**Figure 4**). Using primer set 1, all mice exhibited a band of ~1100 bp except global mGlu5 KO mice, where no band was found in either DRGs or brain (**Figure 4**). WT, floxed mGlu5, and NSmGlu5 mice only had a band at ~1100 bp using primer set 6 (**Figure 4**). All mice used for behavioral experiments described below were verified to not have a band at ~320 bp on genotyping PCR.

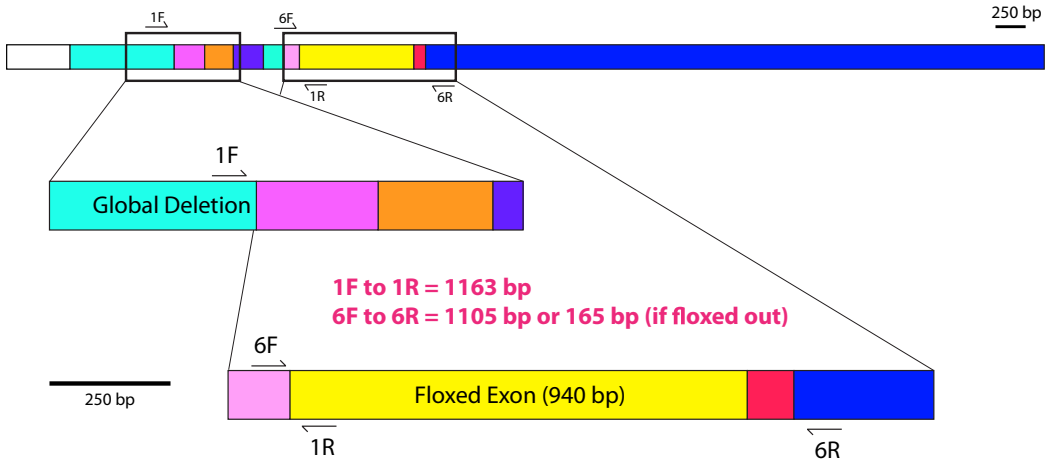

Figure 2: Schematic of mGlu5 cDNA with PCR Primer Binding Sites

A graphical representation of the mGlu5 cDNA along with its UTRs. Each color change represents a different exon. The 5'-UTR is white, the 1st exon is teal, etc. The red box represents the 95 bp addition seen in the b splice variant of mGlu5 (Joly et al., 1995). All exons are to scale. Primer set 1 primers are located in the 1st exon (1F) and the 7th exon (1R), 1163 bp apart. Primer set 6 primers are located in the 6th exon (6F) and 8th exon (6F). The floxed exon is 940 bp, so primer set 6 will produce two different sized products depending on the presence (1105 bp) or absence (165 bp) of exon 7.

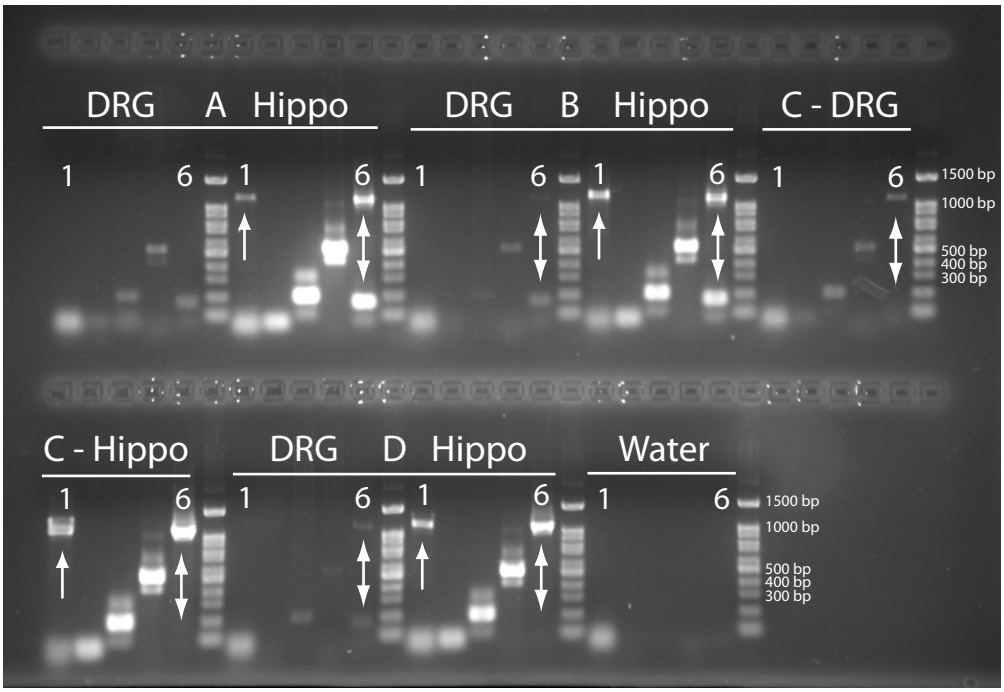

Figure 3: rtPCR of NSmGlu5 and Floxed mGlu5 mice

Representative PCR of cDNA from DRGs and hippocampus of mice with and without the ~320 bp band detected on genotyping PCR. A and B demonstrate the 320 bp band and also show two bands, one at ~160 bp and one at ~1100 bp in both hippocampus and DRGs. C and D did not demonstrate the 320 bp band and hippocampus from these mice only show one band at ~1100 bp. Note that mouse C also did not have the SNS-Cre transgene and is the only mouse not to show a band at ~160 bp in the DRG. Lanes labelled "1" are DNA generated using primer set 1 (single arrow head). Lanes labelled "6" are DNA generated using primer set 6 (double arrow head). Other lanes are generated using primer sets not described.

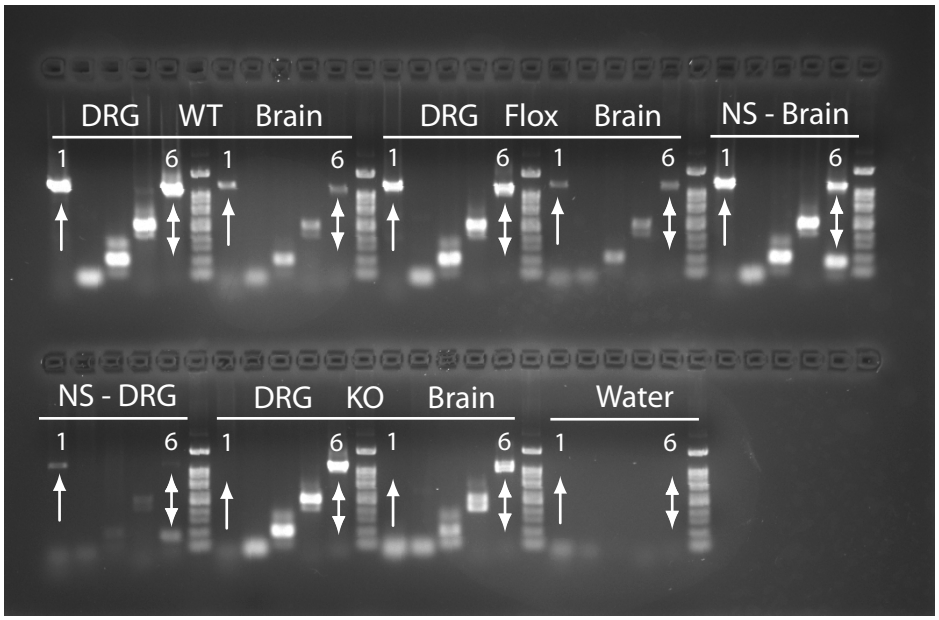

Figure 4: rtPCR of WT, floxed mGlu5, NSmGlu5, and mGlu5 KO mice

Representative PCR of cDNA from DRGs and brain of all mice described in this thesis. The "NS" mouse is a mouse that produced a band at ~320 bp when genotyping for the floxed allele was performed, suggesting that it has an allele that is a global mGlu5 deletion. Note the two bands in the brain of this mouse. All mice, except for the mGlu5 KO mouse exhibited a band at ~1100 bp using Primer Set 1. All mice produced a band at ~1100 bp using primer set 6. Only the "NS" mouse produced a ~160 bp band using primer set 6. Lanes labelled "1" are DNA generated using primer set 1 (single arrow head). Lanes labelled "6" are DNA generated using primer set 6 (double arrow head). Other lanes are generated using primer sets not described.

Peripheral mGlu5 Deletion Does not Affect Open Field Locomotor Activity: NSmGlu5 mice and their floxed littermate controls did not exhibit any difference in the total distance travelled in the open field over a 60 minute period (**Figure 5**).

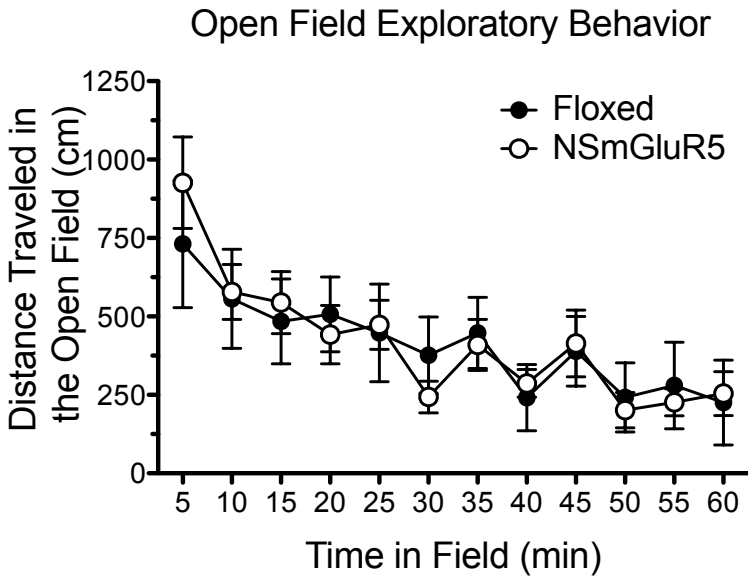

Figure 5: The effects of Conditional mGlu5 Deletion on Open Field Locomotor Behavior

NSmglu5 mice did not differ from floxed littermates when allowed to freely explore an open field for 60 minutes (2-Way ANOVA Main Effect p=0.9106). (n = 4-6 per group.)

Conditional mGlu5 Deletion Does Not Affect Performance on the Accelerating Rotarod: NSmGlu5 KO mice and their littermate controls showed no differences on the accelerating rotarod (**Figure 6**).

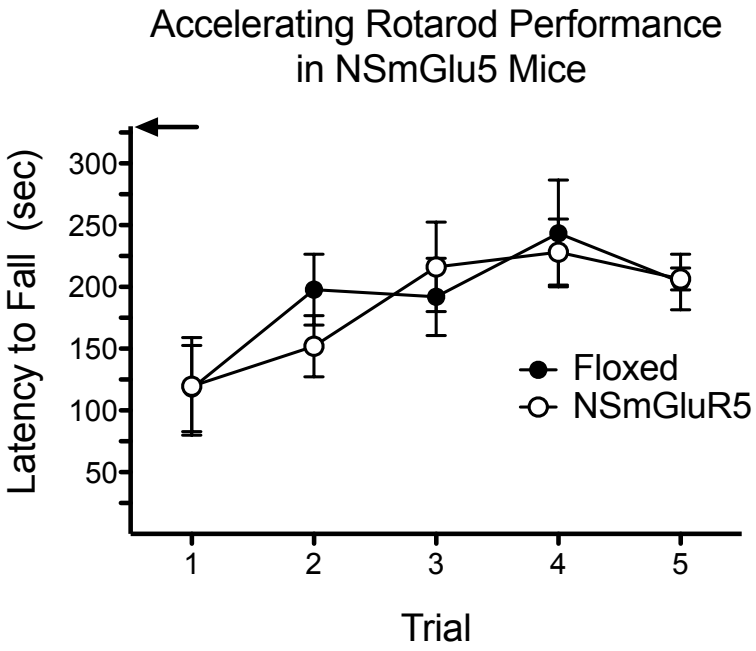

Figure 6: The effects of Conditional mGlu5 Deletion on Accelerating Rotarod Performance

NSmglu5 mice did not differ from floxed littermates in performance on the accelerating rotarod (2-Way ANOVA Main Effect of Genotype p=0.75, n=4-6 per group).

DHPG-Induced Nocifensive Behaviors in NSmGlu5 Mice: Both NSmGlu5 and floxed littermate mice exhibited nocifensive behavior following an intrathecal injection of DHPG. There were no differences noted due to genotype (**Figure 7**).

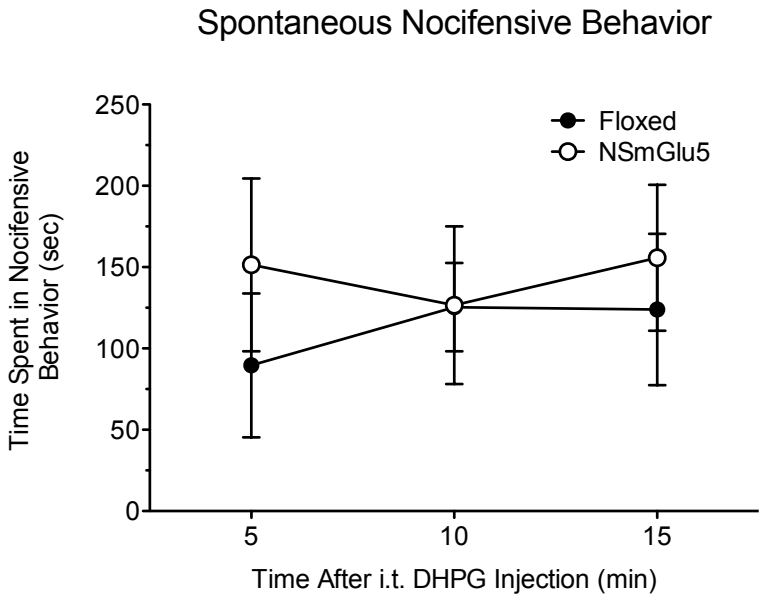

Figure 7: DHPG-Induced Spontaneous Nocifensive Behavior in NSmGlu5 Mice

NSmGlu5 mice did not differ from floxed littermates in time spent in nocifensive behavior following intrathecal DHPG injection (2-Way ANOVA Main Effect of Genotype p=0.3961; n = 4-6 mice per group).

Formalin-Induced Nocifensive Behavior is Not Reduced in NSmGlu5 Mice: Nocifensive behavior, as measured by time spent licking or lifting the injected paw, was not found to be different when NSmGlu5 mice were compared to WT littermates (**Figure 8a-c**).

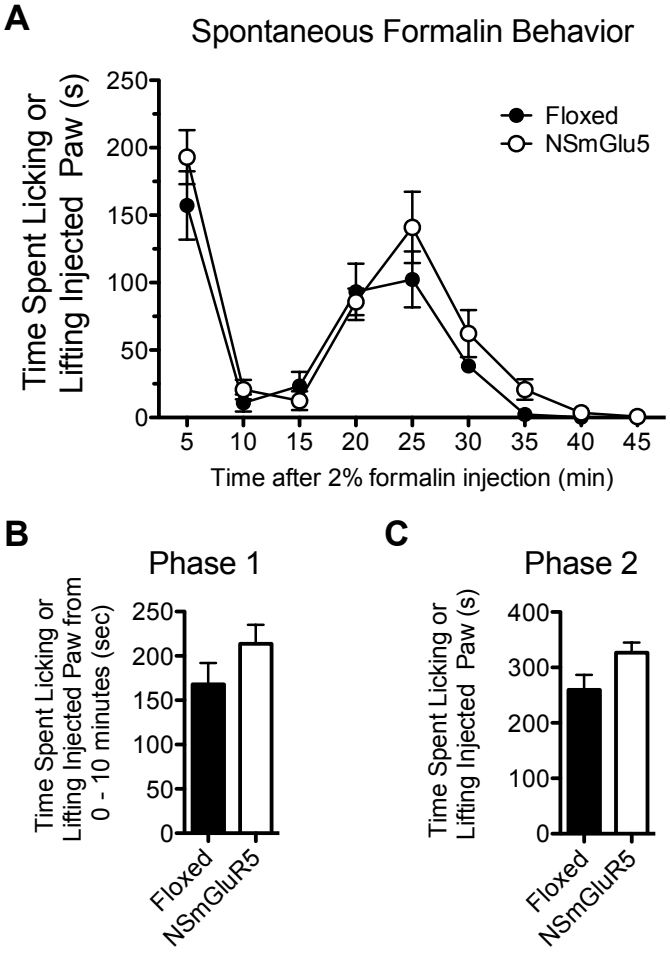

Figure 8: Nocifesnive Behavior Following Intraplantar Formalin Injection in NSmGlu5 Mice.

A) NSmGlu5 and floxed litermates did not differ in their responses to formalin injection (2-Way ANOVA Main Effect of Genotype p=0.0585; n = 4-6 mice per group). The sum of time spent lifting or licking in both B) Phase 1 (0-10 minutes; unpaired t-test p=0.21) and C) Phase 2 (10-60 minutes; unpaired t-test p=0.09) was not different when NSmGlu5 mice were compared to floxed littermates.

DISCUSSION

In chapters 2 and 3 I present data from genetic and pharmacological studies that suggest a role for mGlu5 in the development of pain. However, data presented in Chapter 4 show that targeting mGlu5 for its analgesic potential may also induce increased locomotor behavior and alterations in appetite and food intake, suggesting centrally mediated effects unrelated to analgesia. In this chapter I assessed two pain related behaviors in peripherally restricted conditional mGlu5 KO mice, in which mGlu5 is putatively deleted only in small diameter nociceptive neurons expressing the sodium channel NaV1.8.

NSmGlu5 mice were not found to be different from floxed control mice in two mouse pain models, the formalin test and intrathecal DHPG-induced spontaneous nociception. Both NSmGlu5 and floxed littermate controls exhibited a typical two phase formalin response and were not different from each other either in the first ten minutes (phase 1) or the last 35 (phase 2). In addition, both groups of mice exhibited spontaneous nocifensive behavior following an intrathecal injection of DHPG. When quantified these behaviors were not found to be different between the two groups.

Previous studies have suggested that intrathecal DHPG injection causes spontaneous nocifensive behavior due to direct effects on spinal cord dorsal horn neurons (Karim et al., 2001). In Chapter 2 I presented data that demonstrated a significant reduction in nocifensive behavior following DHPG injection in global mGlu5 KO mice compared to WT littermate controls. NSmGlu5 mice should only have mGlu5 deleted

from peripheral nociceptors and not spinal cord neurons. Thus the finding that deleting mGlu5 only from peripheral nociceptors does not affect DHPG-induced spontaneous behavior, combined with data from the global mGlu5 KO mice presented in Chapter 2, suggests that intrathecal DHPG is primarily exerting its effects on dorsal horn spinal cord neurons or large diameter NaV1.8-negative DRG neurons. While there is some evidence that mGlu5 expressed on the central presynaptic terminals of peripheral nociceptive neurons may contribute to central sensitization by modulating TRPV1 (Kim et al., 2009), the data I present above suggests that presynaptically expressed mGlu5 in NaV1.8-positive neurons does not significantly contribute to spontaneous nocifensive behaviors induced by intrathecal DHPG, at least not at the dose (50 nmols) that I have tested here.

The finding that NSmGlu5 mice do not have significantly reduced nociceptive behaviors in the formalin test is somewhat surprising. Peripheral injection of MPEP reduces formalin-induced spontaneous nociceptive behaviors only when injected into the ipsilateral and not the contralateral paw (Bhave et al., 2001), suggesting a peripheral mechanism of action. This result was taken to suggest that peripherally expressed mGlu5 might be required for the full expression of pain and it was expected that NSmGlu5 mice would exhibit reduced responses to formalin. However, the data from the NSmGlu5 mice presented in this chapter suggests that peripheral mGlu5 in NaV1.8-positive neurons is not necessary for formalin induced spontaneous nocifensive behavior. It is quite possible that peripheral mGlu5 plays a more limited role in nociception than that suggested by previous antagonist studies. It should also be noted that the NSmGlu5 mice tested here

should lack peripheral mGlu5 following functional Cre-Recombinase expression at P0 (Agarwal et al., 2004) and the possibility of compensation due to a lifetime of an absence of peripheral mGlu5 could possibly account for the lack of an effect on the formalin test. However I have no direct evidence to suggest that compensation is occurring here, and as such the most parsimonious explanation for the formalin test findings presented above is that peripheral mGlu5 plays a more limited role in formalin-induced nociception than was previously thought. This may suggest a more limited role for peripheral mGlu5 in other pain-related behaviors as well.

Compensation is but one peril of using genetically modified organisms. When using a Cre-Recombinase / LoxP strategy to excise a gene in a tissue-specific manner any deviation from the expected Cre-recombinase expression pattern may result in a misinterpretation of the results. This is specifically illustrated in the findings presented above that some NSmGlu5 mice exhibit Cre-mediated excision of the 7th exon of mGlu5 in tissues besides the DRGs. Cre transgenes can effect excision of floxed gene sequences in the germline if the Cre-allele and floxed allele are inherited from the same parent (Schmidt-Supprian and Rajewsky, 2007). Unfortunately, due to the desire to avoid testing mice globally heterozygous for mGlu5, the breeding strategy that we chose to generate the NSmGlu5 mice explicitly uses parents that express both alleles. Thus, it is likely that the mGlu5 excision I have found in the brain may be due to cre-mediated excision of mGlu5 in the gametes. The data presented above regarding the locomotor, rotarod, and pain-related behaviors of NSmGlu5 mice use only mice that do not demonstrate a

genotyping PCR band at ~320 bp. This would suggest that they do not have a germline excision of mGlu5, however Cre-fidelity in other tissues has not been directly assessed. Thus, it is also possible that the lack of a difference between NSmGlu5 mice and their floxed littermates is due to undetected ectopic Cre-expression which could result in a mosaic expression of mGlu5. Future users of the NaV1.8 Cre mice developed by Agarwal and Kuner will have to take care to check for cre-mediated excision of their target in unexpected tissue. It may be advisable that future experiments designed to test the effects of peripheral mGlu5 in nociception use a different line of mice in which Cre expression is driven by a knock-in to the NaV1.8 locus (Stirling et al., 2005). A breeding strategy in which mGlu5 het / Cre mice were crossed with homozygous mGlu5 floxed animals would keep the Cre- and floxed- alleles from being inherited from the same parent. However half of the offspring generated in this way would be globally heterozygous for mGlu5, which could potentially confound the interpretation of behavioral experiments.

Overall the data presented in this chapter indicate that peripheral mGlu5 may not be as encouraging a target for the treatment of pain as was previously hoped. However, to paraphrase Bud Craig from the introduction, a mouse is not a human. Indeed there are well known examples of the failed translation into human patients of analgesics predicted to be efficacious by animal models (Mogil et al., 2010). The converse of that axiom is that failed studies in mice do not necessitate failure in man. While these data presented above would not advocate for the rational design of peripherally restricted mGlu5

antagonists as analgesics, should serendipity provide one (for example see Porter et al., 2005), the testing of its analgesic properties would be warranted.

Chapter 6

Summary and Future Directions

mGlu5 is Required for the Full Expression of Pain-Related Behaviors

Overall, the findings presented in this thesis strongly support a role for mGlu5 in the development of pain and suggest that antagonism of mGlu5 may represent a viable treatment option for human pain conditions. As presented in Chapter 2, intrathecal and intraplantar injections of the group I mGluR agonist DHPG induce significantly decreased nociceptive responses and hypersensitivity in mGlu5 KO mice, suggesting that DHPG is exerting its pro-algesic effects by activating mGlu5. In addition, mGlu5 KO mice have decreased spontaneous nocifensive responses induced by intraplantar injection of formalin. Furthermore, when compared to WT littermates, these mice exhibit a faster recovery to baseline mechanical thresholds following injection of the inflammatory agent CFA.

In Chapter 3 I demonstrate that the mGlu5 negative allosteric modulator fenobam reduces nocifensive behavior in WT mice. In addition, fenobam is found to be without analgesic effect in the formalin test when administered to mGlu5 KO mice, suggesting that fenobam's analgesic properties are due to antagonism of mGlu5. These results suggest that mGlu5 is required for the full expression of pain, and imply that allosteric modulation of mGlu5 may have therapeutic effect in the treatment of pain in humans.

As discussed above, mGlu5 is expressed throughout the pain neuraxis. One of the goals of this thesis was to determine the role that mGlu5 expressed in peripheral nociceptive neurons plays in pain. In Chapter 5 I assessed the pain-related behaviors of mice in which mGlu5 had been deleted exclusively from peripheral nociceptors. In

contrast to the global mGlu5 KO mice, pain-related behaviors were not found to be altered in peripherally restricted mGlu5 KO mice, suggesting that peripheral mGlu5 expressed in C-fiber nociceptors is not required for the development of pain. This finding also implies that mGlu5 expressed within the central nervous system or large diameter DRG neurons plays an important role in pain.

The finding presented in Chapter 2 that spontaneous nociceptive behaviors induced by intrathecally injected DHPG are reduced in mGlu5 KO mice implies that activation of mGlu5 within the spinal cord contributes to the development of pain. Additionally, important roles for mGlu5 in pain have also been suggested within the amygdala, and specifically within the laterocapsular division of the central nucleus (CeLC), which receives pain projection neurons from the spino-parabrachio-amygdaloid pathway. Multireceptive (MR) neurons within the CeLC respond to low-intensity stimuli, but are more robustly activated by noxious stimuli. DHPG-induced activation of group I mGluRs within the CeLC potentiates the responsiveness of amygdala MR neurons to innocuous and noxious stimuli both in naive animals and following kaolin/carrageenan-induced arthritis (Ji & Neugebauer, 2010; Li & Neugebauer, 2004). In addition, following arthritis induction, MPEP both inhibits the responses of CeA neurons, and reduces the vocalizations of awake animals following stimulation of the injured knee (Han & Neugebauer, 2005). Administration of DHPG into the CeLC is also sufficient to induce mechanical hypersensitivity and injection of MPEP into the CeLC is able to reverse formalin-induced mechanical hypersensitivity (Kolber, et al., 2010). MPEP has been

found to have residual analgesic efficacy in mGlu5 KO mice (Chapter 3), indicating that it may be mediating this effect in an mGlu5 independent manner. However, formalin-induced mechanical hypersensitivity is also significantly reduced by genetic ablation of mGlu5 selectively within the CeLC (Kolber, et al., 2010), suggesting that CeLC expressed mGLu5 is necessary for the development of hypersensitivity. It is therefore possible that the analgesic effects observed following systemic injections of the mGlu5 antagonist fenobam are due to inhibition of both spinal and amygdalar mGlu5. mGlu5 activation within the spinal cord and the CeLC has been shown to activate the intracellular signaling molecule ERK (Karim et al., 2001; Kolber et al., 2010). In turn, ERK activation within the spinal cord and CeLC has been demonstrated to be required for the full behavioral responses to algogenic stimuli (Karim et al., 2001; Carrasquillo & Gereau, 2007). These findings further implicate mGlu5 as an important cellular component of pain modulation within the spinal cord and the amygdala.

The Translation from Pre-clinical Animal Models to Human Trials

While the findings discussed above support the targeting of mGlu5 for the treatment of pain, additional studies also warrant caution in using mGlu5 antagonists as analgesics due to the potential for adverse side effects. Locomotor and learning effects were reported in mice and rats administered analgesic doses of fenobam (Chapter 4 and Jacob et al., 2009). Additionally, findings from mGlu5 KO support a role for mGlu5 in normal locomotor activity and in appetitive behavior and weight gain. mGlu5 KO mice were found to have significantly higher locomotor exploratory behavior when compared to WT mice and the mGlu5 antagonist fenobam increased locomotor behavior in WT mice compared to vehicle injected mice. In addition, fenobam significantly suppressed food intake following a 24 hour fasting period, suggesting that fenobam may decrease appetite.

However, mGlu5 KO mice were found to be indistinguishable from their WT littermates in motor coordination tasks. Also, an analgesic dose of fenobam did not affect motor coordination assessed using an accelerating rotarod. While the alterations in locomotor behavior and food-intake are clearly off-target, they may not sufficiently deleterious to prevent the use of fenobam as an analgesic. Finally, no side-effects were reported in 3 healthy human volunteers orally administered 150 mg of the fenobam (Berry-Kravis et al., 2009), suggesting that the analgesic effects of fenobam may be ready for assessment in human subjects.

Fenobam is not the only mGlu5 antagonist that could potentially be tested in

humans, and several drug companies have lead compound mGlu5 antagonists in early clinical testing. For example, Addex Pharmaceuticals has begun conducting trials of one of its lead mGlu5 NAM compounds, ADX10059, and has reported efficacy as a treatment for migraine in a phase 2A trial (Keywood, 2008). ADX10059 (750 mg total dose) was reported to have a good safety profile in a separate trial examining its utility as a treatment for gastro-esophageal reflux disease (Keywood et al., 2009). However, a phase 2B trial involving cumulative doses of ADX10059 (up to 200 mg per day) has been recently terminated due to a higher than expected incidence of abnormal liver functions tests (ClinicalTrials.gov ID: NCT00820105, accessed March 3, 2010). While the data presented in this thesis suggests that mGlu5 antagonists may have utility in the treatment of chronic pain conditions, clearly carefully conducted future testing in humans will be necessary to ascertain whether potential analgesic effects are separable from dose-limiting side effects.

In addition to treating migraine, mGlu5 antagonists may have a role in the treatment of other pain conditions, such as post-operative pain, arthritis, and fibromyalgia. mGlu5 antagonists may be useful as mono-therapies, or in combination. For example, it is possible that ligands acting at mGluRs may be useful as adjuvants for opiate analgesia. NMDA receptor antagonists are known to attenuate the development of morphine tolerance (Marek et al., 1991) and several studies have indicated that ligands for group I and II mGluRs may have similar effects. Systemic administration of the mGlu5 antagonist MPEP and the group II mGluR agonist LY379268 have been shown to

improve the analgesic efficacy of morphine and reduce the development of tolerance in a model of neuropathic pain (Kozela et al., 2003; Osikowicz et al., 2008). Further studies will be necessary to expand upon these initial studies and to determine whether mGluR ligands may act as adjuvants for other known analgesics, such as non-steroidal anti-inflammatory agents.

The Heat/Capsaicin Trial as a Method for Assessing Fenobam as an Analgesic

In addition to conducting clinical trials in patients, human experimental pain models play an important role in the assessment of the analgesic properties of novel compounds. Predictive and non-invasive pain models can facilitate the transition from pre-clinical testing to more expensive clinical trials by providing a cost-effective way to assess analgesic efficacy in a limited number of healthy volunteers. Future studies should be performed that assess the ability of fenobam to modulate human pain in healthy volunteers, thus expanding the pre-clinical data into human subjects.

While substantial pre-clinical data exist indicating that negative allosteric modulators of mGlu5 may mediate their analgesic effects via prevention or reversal of central sensitization in rodents, no findings that directly test this hypothesis in human subjects have been published. One way to address this question would be to test the ability of fenobam to reduce the hypersensitivity caused by the heat/capsaicin experimental hyperalgesia model in healthy human volunteers. The heat/capsaicin model is a safe and non-invasive method that utilizes serial application of heat and capsaicin to synergistically produce stable, long-lasting, and reproducible hyperalgesia and hypersensitivity in human subjects (Dirks, 2003). The application of both heat and capsaicin produces an expanded area of hypersensitivity and allodynia outside of the area of application that is akin to that caused by central sensitization following frank tissue injury. Multiple studies have illustrated that drugs with known analgesic efficacy, including gabapentin (Dirks, 2002) and morphine (Frymoyer, 2007), reduce the

hypersensitivity induced by the heat/capsaicin model. To test directly whether antagonism of mGlu5 can reduce central sensitization in human subjects it would be possible to induce sensitization using the heat/capsaicin model and compare the ability of fenobam to reverse this sensitization relative to placebo in a double-blind crossover study. Performing this study would provide direct evidence of the efficacy of fenobam to reduce hypersensitivity in a validated human experimental model of central sensitization and may provide the rationale for continued development of mGlu5 antagonists as analgesics in humans.

The heat/capsaicin hyperalgesia model combines serial heat stimulation (45º C for 5 min) applied to a 15.7 cm^2 area of skin on the forearm and topical low dose capsaicin (0.1% Capzacin-HP Cream; Chattem, Chattanooga, TN) applied to the same area. This combination produces reversible pain and the sensory changes associated with peripheral and central sensitization for up to 4 hours. Application of thermal stimulations are applied in a precise and controlled manner using a Medoc Advanced Thermal Stimulator (Medoc, Ramat Yishai, Israel) driving a 9 cm^2 thermode. The thermode is a computer-controlled Peltier device that warms the skin from 32º C to a safety cutoff of 50º C in 1º C / s increments. The heat/capsaicin model has been utilized in numerous studies involving human subjects that demonstrate that it is safe and reliable and does not damage skin (Abrams et al., 2007; Dirks et al., 2001, 2002, 2003).

The following methods would be used to induce and quantify pain and central sensitization: A) pain intensity produced by 45º C thermal stimulation for 1 minute, B)

heat pain detection threshold to a 1º C ramp stimulus from 32º C to 50º C, C) the pain intensity and area (in cm^2) of secondary hyperalgesia and allodynia induced by the heat/ capsaicin model.

Central sensitization would be established by heating the volar surface of the dominant forearm using the thermode set to 45º C for 5 minutes, followed by immediate application of approximately 0.5 ounces of 0.1% capsaicin cream covering the previously heated surface (9 cm^2). The cream would be left on for 30 minutes and then wiped off. At the end of the 30 minute capsaicin application, measurements would be performed to determine the areas of hypersensitivity and allodynia on the forearm. The borders of secondary hyperalgesia would be mapped using a 1 inch foam brush and a nylon Von Frey filament (26 g bending force) along four linear paths between the thermode outline and 1) the antecubital fossa , 2) the wrist joint, 3) the lateral aspect of the forearm in anatomical position, and 4) the medial aspect of the forearm (**Figure 1**).

Hypersensitivity would be maintained by "rekindling" the site of heat/capsaicin application by re-stimulating the previously treated skin four times at 40 minute intervals with the thermode set to a lower temperature (40º C) for 5 min. Rekindling has been shown to maintain robust and consistent hypersensitivity in human subjects for at least 4 hours (Dirks, 2003).

It would be possible to study the effects of fenobam on the hypersensitivity caused by the heat/capsaicin model by calculating the change from baseline hypersensitivity due to the effects of fenobam and comparing it to an inert placebo. Baseline would be defined

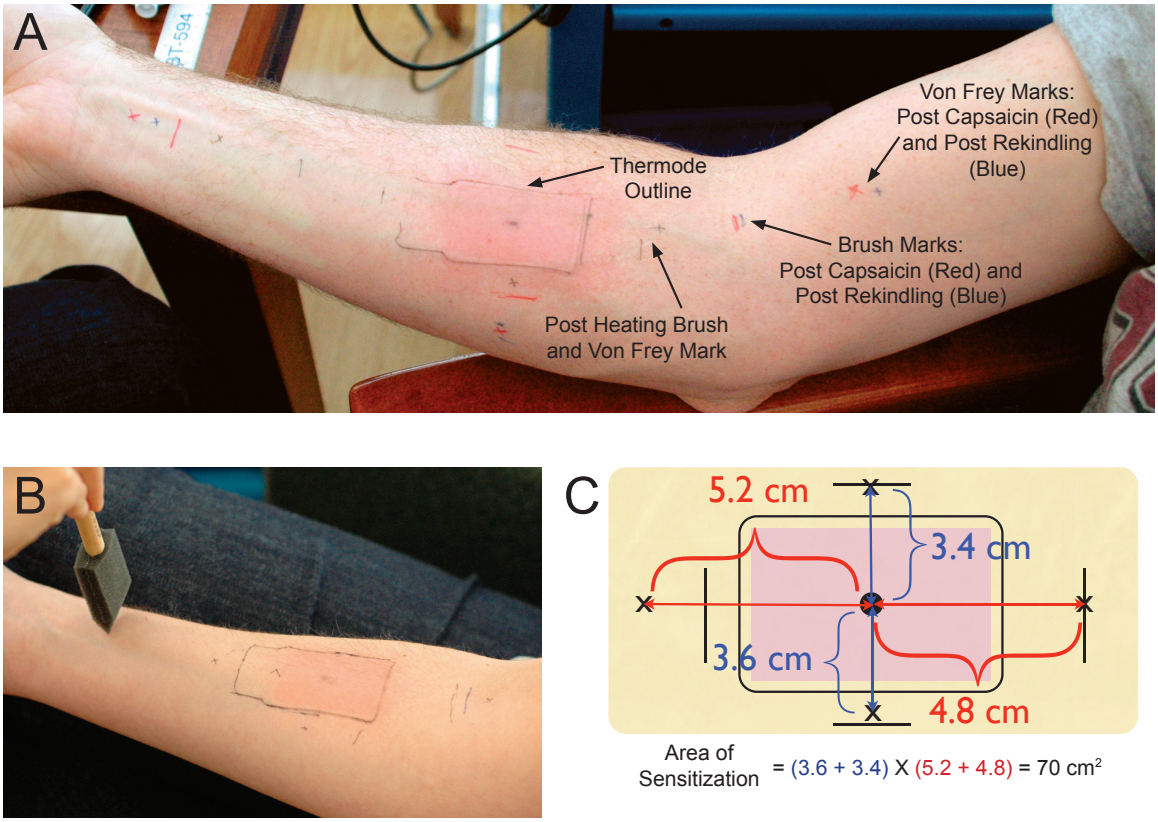

Figure 1: The Heat/Capsaicin Model.

A) Photograph of a subject's forearm immediately following the first rekindling. Note the erythema within and around the thermode outline. Marked lines indicate locations where stimulation with a foam brush was perceived as painful. Hash marks indicate locations where stimulation with a 26 g Von Frey hair was perceived as painful. Black, red, and blue marks indicate measurements performed following the 5 min 45º C heating, the 30 minute capsaicin application, and the first rekindling (5 min at 40º C) respectively. B) Use of the foam brush to map out the area of hypersensitivity following capsaicin cream application. C) Diagram demonstrating how the area of hypersensitivity from 4 hypothetical Von Frey measurements (X marks) would be calculated.

as the size of the area of hypersensitivity immediately following the first rekindling. After the first rekindling either fenobam (150 mg per os) or placebo would be administered. Three post-drug rekindling sessions would then be performed. After the third post-drug rekindling procedure the area of the hypersensitivity would be mapped and any change

from baseline hypersensitivity would be determined. In addition, it would be possible to quantify the fenobam plasma concentration by performing a blood draw on the volunteers and then performing liquid chromatography-mass spectrometry on the plasma as described in Chapter 3. Finally, assessment for tolerability and safety following fenobam administration would be readily comparable to inert placebo.

Based on previously published findings assessing the effects of orally administered fenobam in healthy volunteers (Berry-Kravis, 2009), it is expected that fenobam at doses up to 150 mg will be well tolerated with minimal side effects. It is also anticipated that fenobam administration will reduce the heat/capsaicin-induced area of hypersensitivity on the forearm, as compared to placebo. Fenobam is also expected to reduce the pain intensity during rekindling when compared to placebo. These findings would indicate that fenobam is capable of reducing central sensitization and suggest a prominent role for mGlu5 in the maintenance of central sensitization in human subjects. It would also encourage the testing of the analgesic efficacy of fenobam in human pain patients.

In conclusion the work in this thesis supports the development of mGlu5 antagonists as analgesics, provided that care is taken in the assessment for clinically significant deleterious effects.

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Curriculum Vitae

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University of Virginia Undergraduate Research Fellowship (2002)

Intel Science Talent Search National Semifinalist (1999)

CONFERENCE PUBLICATIONS

Montana MC, Zhao CS, Morales ME, Xu J, Heinemann SF, Agarwal N, Kuner R, & Gereau RW 4th (2010). "The role of peripheral mGlu5 in pain behavior." Society for Neuroscience Annual Meeting in San Diego, CA, November 13-17, 2010 (Poster # 81.12/VV10).

Chiechio S, Zammataro M, **Montana MC**, Copani A, Nicoletti F, & Gereau RW 4th (2010). "mGlu2 and mGlu3 receptors differently modulate inflammatory pain in mice." Society for Neuroscience Annual Meeting in San Diego, CA, November 13-17, 2010 (Poster # 81.18/VV16).

Montana MC & Gereau RW 4th (2009). "Analysis of pain behaviors in mGlu5 knockout mice." Society for Neuroscience Annual Meeting in Chicago, IL, October 17-21, 2009 (Poster # 764.17/CC57).

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Montana MC, Cavallone, LF, & Gereau RW 4th (2008). "The metabotropic glutamate receptor 5 (mGlu5) antagonist fenobam is analgesic in rodents." Society for Neuroscience Annual Meeting in Washington, DC, November 15-19, 2008. (Poster # 772.5/MM20).

PUBLICATIONS

JOURNAL ARTICLES

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